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MOLECULAR BIOLOGICAL STUDIES ON THE BIOGENESIS OF HUMAN CHOLINESTERASES IN VIVO AND AS DIRECTED BY CLONED CHOLINESTERASE DNA SEQUENCES

MIDTERM REPORT

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In order to study the multileveled regulation of the human cholinesterase genes and their protein products, the cDNAs encoding for butyrylcholinesterase and acetylcholinesterase in several human tissues were isolated and compared to each other and to other cDNAs encoding related proteins. Xenopus oocytes were used to express the synthetic mRNA for butyrylcholinesterase alone of with tissue mRNAs. Cross-homologies and structural differences between cholinesterases were revealed by antibodies against bacterially produced butyrylcholinesterase polypeptides, which also react with the homologous thyroglobulin. In situ hybridization was employed to reveal the expression of cholinesterase mRNA in human oocytes. Amplification of the CHE gene was found in a family exposed to parathion as well as in blood cells from patients with leukemias or platelet disorders.

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Summary

Overview: This report is focused on studies directed at revealing the multileveled regulation of the human genes coding for cholinesterases (ChEs), their mRNA and protein products and their in vivo mode of expression and alteration. ChEs present an intriguing diversity of molecular structures, cellular localizations, and possibly functions. Besides their intrinsic physiological importance as acetylcholine-hydrolyzing enzymes, ChEs constitute a privileged model for exploring a variety of genetic, transcriptional and post-transcriptional mechanisms generating a complex array of molecular structures. During the recent 18 months we have combined various approaches to study this interesting gene family, as is summarized in the following.

Molecular cloning of human acetylcholinesterase cDNA from various tissue origins: To isolate the cDNAs encoding human nervous system acetylcholinesterase (AChE), oligodeoxynucleotide probes were synthesized according to the amino acid sequences in evolutionarily conserved and divergent peptides from electric fish AChE as compared with parallel regions in human butyrylcholinesterase (BuChE), isolated under previous contracts. Comparative screening of cDNA libraries from fetal human basal brain nuclei, brainstem, liver and muscle resulted in the isolation of several cDNA clones encoding a polypeptide with >50% homologies to both Torpedo AChE and human BuChE, strongly suggesting that it is human AChE. DNA sequencing displayed a limited nucleotide sequence similarity with BuChEcDNA. AChEcDNA, isolated under the present contract, was relatively abundant in the basal brain nuclei library, whereas BuChEcDNA was found to be more abundant in liver. DNA blot hybridization has further shown that AChEcDNA hybridizes with a limited number of human genomic DNA fragments, different from those hybridizing with BuChEcDNA. These findings suggest that in humans, the highly homologous AChE and BuChE are encoded by different genomic sequences, each of which might be subjected to distinct tissue-specific regulatory signals.

Similar butyrylcholinesterase mRNA transcripts in fetal and adult liver: Screening of cDNA libraries from adult liver and lymphocytes resulted in the isolation of 2500-nucleotide-long cDNA clones which, when sequenced, were found to be identical with the previously isolated clones from fetal brain and liver origins. This analysis demonstrated that a similar mechanism of post-transcriptional processing leads to the production of active butyrylcholinesterase in fetal and adult liver, as well as in lymphocytes, and strongly suggested that a single gene codes for this enzyme in fetal and adult tissues.

Post-translational regulation: To examine the involvement of tissue-specific elements in the processing and compartmentalization of the ubiquitous, polymorphic ChE proteins, the expression of BuChE was analyzed in Xenopus oocytes microinjected with synthetic BuChEmRNA alone and in combination with tissue-extracted mRNAs. When injected alone, BuChEmRNA efficiently directed the synthesis of primarily dimeric molecules which formed small membrane-associated accumulations principally localized on the external surface of the oocyte's animal pole. Co-injection with brain or muscle mRNA generated additional

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tissue-specific BuChE molecular forms and facilitated the formation of pronounced, similarly segregated tissue-characteristic BuChE aggregates. These findings implicate tissue-specific mRNAs in the assembly of the clone-produced protein as well as in its non-uniform distribution in the oocyte membrane or extracellular material.

Cross-immunoreactivity of nascent peptides: In order to study the polymorphism of human ChEs at the levels of primary sequence and three-dimensional structure, a fragment of human BuChEcDNA was subcloned into the pEX bacterial expression vector and its polypeptide product was analyzed. Immunoblot analysis revealed that the clone-produced BuChE peptides interact specifically with antibodies against human and Torpedo AChE. Rabbit polyclonal antibodies prepared against the purified clone-produced BuChE polypeptides interacted in immunoblots with denatured serum BuChE as well as with purified and denatured erythrocyte AChE. In contrast, native BuChE tetramers from human serum, but not AChE dimers from erythrocytes, interacted with these antibodies in solution to produce antibody-enzyme complexes which could be precipitated by second antibodies and which sedimented faster than the native enzyme in sucrose gradient centrifugation. Furthermore, both AChE and BuChE dimers from muscle extracts, but not BuChE tetramers from muscle, interacted with these antibodies.

Autoimmune response in hyperthyroidism: The nature of the putative autoantigen in Graves' ophthalmopathy (GO) remains an enigma but the sequence homology between thyroglobulin (Tg) and ChE provides a rationale for epitopes which are common to the thyroid gland and the eye orbit. Using recombinant human BuChE polypeptides representing the region presenting high homology with Tg and conventionally prepared human Tg in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we have demonstrated the binding of rabbit and human antibodies to Tg and rabbit anti-cloned BuChE to both proteins. Subsequently immunoglobulins (Igs) from 7/9 patients were shown to bind to the ChE proteins in dot blot and electrophoretic protein blot experiments in which 5 normal IgGs were negative. This was confirmed by in situ immunolocalization in which Igs from 6 GO patients tested were shown to bind to end plate regions of human fetal muscle fibers which were concurrently shown to be cytochemically rich in ChE activity; 3 normal Igs did not bind. The results demonstrate that cross-reactivity between antibodies to Tg and ChE exists in vivo and that Igs from GO patients contain antibodies which bind to ChEs. These findings may bear significance in the pathogenesis of GO.

Expression of cholinesterase genes in human oocytes revealed by in-situ hybridization: Transcriptional activity of the human ChE genes was examined in developing oocytes from mature ovaries by in-situ hybridization combined with biochemical acetylcholine hydrolysis measurements. High levels of BuChEmRNA could be detected in oocytes from primordial, pre-antral and antral follicles but not in atretic follicles, with transient enhancement at the pre-antral stage. In view of the very limited sequence homology between BuChEcDNA and AChEcDNA, this analysis reflects selective production of BuChEmRNA transcripts in the developing oocytes. Our findings suggest that cholinergic responses may function in human oocytes independently of the surrounding

follicular cells, and the pronounced synthesis of BuChEmRNA transcripts in oocytes suggests that the ChE genes in humans are particularly good candidates for the formation and re-insertion of inheritably amplified ChE genes.

Gene amplification: Mammalian cell cultures and tumor tissues often acquire resistance to drugs or inhibitors by selection of cells with inheritable amplified genes encoding the target protein of these agents. Well-known examples are the amplified dihydrofolate reductase, Na,K-ATPase and ribonucleotide reductase genes, conferring resistance to methotrexate, ouabain or hydroxyurea, respectively. Numerous DNA amplification events were found in primary and metastatic tumors and in established cell lines, but not in whole animals or in humans. During the course of this work, we found a de novo 100-fold amplification of a genomic DNA fragment hybridizing with BuChEcDNA and localized it onto the long arm of chromosome No. 3. The amplification occurred in an individual under prolonged exposure to organophosphorous insecticides who displays an unusual serum BuChE phenotype. A similar amplification was found in a son of this individual. These findings present a novel example of an inheritable amplification within an autosomal gene in apparently healthy individuals and imply that the frequent use of organophosphorous poisons may have long-term inheritable consequences in humans.

Co-amplification of human acetylcholinesterase and butyryl-cholinesterase genes in blood cells: In order to study the yet unknown role of the ubiquitous family of ChEs in developing blood cells, AChE-cDNA and BuChEcDNA were used in blot hybridization with peripheral blood DNA from various leukemic patients. Hybridization signals intensified 10-200-fold and modified restriction patterns were observed with both cDNA probes in 4 out of the 16 leukemia DNA preparations examined. These reflected the amplification of the corresponding ACHE and CHE genes, encoding AChE and BuChE, respectively, and alteration in their structures. Parallel analysis of 30 control samples revealed non-polymorphic, much weaker hybridization signals for each of the probes. In view of previous reports on the effect of acetylcholine analogues and ChE inhibitors in the induction of megakaryocytopoiesis and production of platelets in the mouse, we further searched for such phenomena in non-leukemic patients with platelet production disorders. Amplifications of both ACHE and CHE genes were found in 2 of the 4 patients so far examined. Pronounced co-amplification of these two related but distinct genes in correlation with pathological production of blood cells suggests a functional role for members of the ChE family in megakaryocytopoiesis, and raises the question whether the co-amplification of these genes could be causally involved in the etiology of hemocytopoietic disorders.

FOREWORD

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DATE

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Table of Contents

List of Figures and Tables

List of Abbreviations

ACh	Acetylcholine.
AChE	Acetylcholinesterase.
ACHE	Gene coding for AChE.
AML	Acute myelodysplastic leukemia.
BuChE	Butyrylcholinesterase.
BuSCh	Butyrylthiocholine.
BW284C51	1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide.
cDNA	Complementary deoxyribonucleic acid.
ChE	Cholinesterase.
CHE	Gene coding for BuChE.
DFP	Diisopropylfluorophosphate.
GO	Graves' Ophthalmopathy.
Igs	Immunoglobulins.
iso-OMPA	tetraisopropylpyrophosphoramide.
mRNA	Messenger ribonucleic acid.
OP	Organophosphorous.
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tg	Thyroglobulin.

I. Introduction: Multileveled regulation of the human cholinesterase genes and their protein products

I.1. Overview and general significance

The family of cholinesterases (ChEs) has been the subject of intensive research for five decades, with a continuous increase in the number of studies being focused on this family of enzymes as well as in their scope and diversity. At the biochemical level, ChEs are highly polymorphic carboxylesterases of broad substrate specificity. It has been assumed for a long time that ChEs are involved in the termination of neurotransmission in cholinergic synapses and neuromuscular junctions. According to the accepted classification of enzymes, ChEs belong to the B type carboxylesterases on the basis of their sensitivity to inhibition by organophosphorous (OP) poisons (1). ChEs are primarily classified into acetylcholinesterase (AChE, acetylcholine acetyl hydrolase, EC 3.1.1.7) and butyrylcholinesterase (BuChE, acylcholine acylhydrolase, EC 3.1.1.8) (2), according to their substrate specificities and sensitivities to selective inhibitors However, the complete scheme is much more complex. Further classifications of ChEs are based on their electrical charges, their levels of hydrophobicity, the extents and modes of their interactions with membrane or extracellular structures and, last but not least, the multisubunit association of catalytic and non-catalytic "tail" subunits composing together the biologically active ChE molecules (3-5).

Numerous studies over the years have indicated that the severe clinical symptoms resulting from intoxication by OP agents (6) are caused by their very tight, irreversible inhibitory interaction with ChEs (7,8). OPs are substrate analogues to ChEs, which display impressive dissociation constants with their catalytic subunits. The labeled OP diisopropylfluorophosphate (DFP) was shown to bind covalently to the serine residue at the active esteratic B site region of ChEs, which is common to all of the carboxylesterases (9,10). This property has been used in research aimed at protein-sequencing studies. The binding and inactivation capacities of OPs on ChEs are considerably higher than their effects on other serine hydrolases. Furthermore, even within species the inhibition of specific ChEs by different OPs tends to be highly selective to particular ChE types (11). In order to improve the designing of therapeutic and/or prophylactic drugs to the short- and long-term effects of OP intoxication, it is therefore desirable to reveal and compare the primary amino acid sequence and three-dimensional structure of all of the members belonging to this enzyme family, as well as to the homologous domains in other serine hydrolases. Elucidation of these sequences and their interactions within the ChE molecule and with other elements can deepen the understanding of the mode of functioning of ChEs and the specific amino acid residues involved in this functioning. This has therefore been one of the main directions of our research. In the following, we report the analysis of sequence similarities between human AChE and related proteins, based on molecular cloning, DNA sequencing and computer analysis of the derived sequences. This analysis revealed, within the period covered by this report, quite unexpectedly, that the cDNA sequences encoding AChE and BuChE do not display a high sequence homology, in spite of the considerable similarity between the protein sequences encoded by these two cDNAs.

I.2. Molecular form heterogeneity in cholinesterases

ChEs constitute a family of carboxylesterase type B enzymes, all of which catalyze the hydrolysis of choline ester compounds at high rates. The localization of such enzymes at cholinergic synapses, where acetylcholine (ACh) is released and must be rapidly inactivated, can be expected. The enzyme present and concentrated at the neuromuscular junction (12,13), AChE exhibits a high affinity for the neurotransmitter ACh and is named after this affinity, acetylcholinesterase. In addition to AChE it has been demonstrated that a high activity of butyrylcholine-hydrolyzing enzyme was present both in the serum (14) and in cholinergic synapses (15). This enzyme, named butyrylcholinesterase (BuChE), is also expressed in many additional cell types, including multiple types of embryonic and tumor cells as well as hepatocytes, muscle fibers, endothelial cells and lymphocytes (16). The role of this enzyme in all of these sites is completely unknown. In addition to ACh, it is capable of hydrolyzing chemically related substrates such as succicurarium, various local anesthetics based on choline esters and related compounds (1,17).

In the seventies, different groups (18,19) studied in detail the molecular polymorphism of both AChE and BuChE in various species, using a variety of biochemical techniques. Several conclusions emerged from this series of studies.

a) The various globular molecular forms of AChEs and BuChEs include the monomeric, the dimeric and the tetrameric oligomers of catalytic subunits and are principally composed of catalytic subunits alone.

b) The group defined as "asymmetric" molecular forms of AChEs includes one triple-helical, collagen-like, non-catalytic "tail" subunit associated with one, two or three catalytic tetramers. The collagen-like "tail" consists of long (50-nm) fibrillary peptides, rich in proline and hydroxyproline residues (20), and is generally assumed to serve as an attachment of its associated tetramers to solid matrix, for example, that of the extracellular basal lamina. The same research groups were also able to demonstrate the physical buildup of these enzymes at the electron microscopic level, supporting the hypothesis of attachment through the non-catalytic "tail" subunits. The above model corresponds primarily to the molecular forms of AChEs observed in the electric fishes, namely Torpedo marmorata and the electric eel Electrophorus electricus (21). The electric organ of these fishes exhibits very high concentrations of both the nicotinic ACh receptor and of AChE, which is why it served as a highly appropriate model system for studies directed at these molecules. However, it should be noted that the sedimentation coefficients observed for the enzyme in insects, amphibians (22), avians (23), and mammals (15,24,25) indicate different molecular weights and/or distinct compositions of catalytic and non-catalytic subunits from those previously reported for the Torpedo enzyme.

In addition to AChEs, the above model can be applied for the molecular forms of BuChE, which have very similar structures and sedimentation coefficients as compared with AChE (26,27). Recently, a new type of enzyme was described, in embryonic chicken

cells, which contains hybrid tetramers associating two dimers of BuChE with two others composed of AchE subunits (28). This adds a new dimension to the accepted scheme of molecular forms; It also increases the expected level of complexity of molecular polymorphism to be searched for in this enzyme family.

In addition to the different compositions of subunits, the molecular polymorphism generally corresponds to different developmental alterations, tissue specificities, and subcellular localizations. The enzyme can be either soluble in the cytoplasm, bound to membrane structures or associated with the extracellular matrix material, all according to its tissue origin, and the mechanisms responsible for this complex heterogeneity have not been pursued so far.

It has been demonstrated that the monomeric and dimeric forms of AChEs are generally bound to the plasmatic membrane (29) or to the endoplasmic reticulum, through a phospholipid link which binds a hydrophobic domain of the protein (30-32). In addition, the same molecular forms of the enzyme are present as cytoplasmic soluble proteins. The higher oligomeric forms, which mainly appear as ectoenzymes, are bound to the plasmatic membrane through a highly hydrophobic 20-kDa subunit composed of lipid components that has been found in the bovine brain (33). The tetrameric enzyme can also be low salt soluble as in the cerebrospinal fluid, where it is the major AChE form, and in the serum, where the soluble BuChE tetramer is predominant. Nothing is known as yet regarding the mode of attachment of BuChE catalytic subunits to solid support.

The asymmetrical forms of AChE are assumed to be associated with the fibrillar molecules of the basal lamina (15,34) through numerous ionic bonds. This assumption is supported by the observation that these tailed forms become soluble at high ionic strength (e.g., 1M NaCl or 0.5M $MgCl^{2+}$) and may be dissociated from their solid support and kept in solution. A small proportion (ca 20%) of the asymmetrical form remains associated to lipidic membranes even in the presence of salt and may be solubilized by the addition of detergent (32). Several extensive reviews of the molecular forms' heterogeneity in ChEs have appeared (2,35,36).

In humans, the ChEs in different tissues also exhibit a high degree of polymorphism, as each expresses a different pattern of molecular forms. In the liver, the monomeric and dimeric forms of BuChE are detectable and predominant (Soreq et al., unpublished data). The external surface of the red cell has long been known to be extremely rich in the AChE dimeric form (37), and to a lesser extent in the monomeric form (38). In the human fetal brain, the main form is a membrane-bound, amphiphilic tetrameric AChE (25). This form represents about 90% of the total activity, excluding the serum activity. It is in majority bound to the external surface of the neurons. A small amount of 16S has been detected, but it represents only 1-2% of the activity (39). BuChE (40), mainly as tetramer, has also been demonstrated (25). The cerebro-spinal fluid is very rich in soluble 10S tetrameric AChE, which is probably secreted by the neurons (41). In the spinal cord the amphiphilic 10S AChE tetramer is predominant (Dreyfus et al., unpublished data).

In the neurons of the peripheral autonomous nervous system, all the molecular forms of both AChE and BuChE are more or less detectable (42). This is also the case in the muscle fiber, where the cholinesterases are mainly concentrated at the neuromuscular junction (15) and at the myotendinous junctions.

I.3. Microinjected Xenopus oocytes as a heterologous expression system to study cholinesterase biosynthesis

Xenopus laevis oocytes have proven a valuable in vivo expression system for the production of a variety of biologically active membrane proteins from synthetic and tissue-derived mRNAs (47). Proteins recently studied in the oocyte system include the ACh receptor (48,49), peptide and amino acid neurotransmitter receptors (50), and various channel proteins (51). In advanced studies, Xenopus oocytes have been used in conjunction with site-directed mutagenesis to investigate structure-function relationships in specific polypeptides.

In order to approach the molecular mechanisms underlying the biogenesis of ChE's, a full-length cDNA clone coding for human BuChE (43) was subcloned, during the period covered by this report, into the SP6 transcription vector (44). Synthetic polyadenylated BuChEmRNA was transcribed in vitro and microinjected into Xenopus oocytes, where the translation of tissue-extracted ChEmRNAs has previously been demonstrated (45,46). Co-injection of the synthetic BuChEmRNA with total poly(A)$^+$ RNA from brain and muscle was then employed to examine the involvement of additional tissue-specific factors in the assembly and compartmentalization of the enzyme in the oocytes.

I.4. The use of genetic engineering to re-examine the immunochemical cross-reactivity of anti-cholinesterase antibodies

Previous attempts to reveal the molecular origin for the heterogeneity of ChEs were based on the elicitation of polyclonal and monoclonal antibodies against minute quantities of highly purified AChE, prepared from the electric organ of Torpedo (52-54), brain tissue (55-57) or red blood cell membranes (58-60). The antibodies produced interacted with all of the molecular forms of either AChE or BuChE. However, antibodies elicited against AChE did not cross-react with BuChE and vice versa (55,60,61). This was generally interpreted to indicate sequence dissimilarities between AChE and BuChE (62). On the other hand, monoclonal antibodies with significant cross-reactivity have been seen by at least one group (53). Also, recent cDNA cloning (43,62) revealed 53% homology between the amino acid sequence of the human serum enzyme and that of Torpedo AChE (63) including several identical regions of at least 10 successive amino acid residues. This strongly suggests a common ancestral origin for the two enzymes, which could indicate that the lack of immunological cross-reactivity between AChE and BuChE is not due to a lack of sufficient homology but reflects structural differences. For example, distinct folding patterns of the polypeptide chains could mask homologous regions, or particular glycosylation chains

could have the same effect. Another possibility is that the homologous regions are those demonstrating low immunogenicity. To examine these possibilities, one would need to elicit antibodies against specific regions of the nascent polypeptide chain that show the greatest homology between various classes of ChEs, or which display low immunogenicity.

For this purpose, the N-terminal part of the human BuChE protein, which displays the highest sequence homology to Torpedo AChE (amino acid residues 1-198, see ref. 5), was produced in bacteria from an DNA plasmid containing 760 nucleotides from the cloned BuChEcDNA. Antibodies were elicited against this polypeptide and were shown to cross-interact with specific molecular forms of both AChE and BuChE from various human tissues.

I.5. The putative cross-reactivity between antibodies to thyroglobulin and cholinesterases and its clinical implications

Several studies (64,65), have implied that thyroglobulin (Tg), or an immunogenically Tg-like protein, may be the antigen common to both proteins from thyroid and eye orbit. Furthermore, there is evidence that the protein eliciting an immunological response in the orbit muscle is not Tg itself (66). Recent molecular cloning studies revealed a significant homology between the carboxyl terminal of Tg and the N-terminal half of Torpedo AChE (63) and human BuChE (43). Furthermore, a comparison of their hydropathy profiles (5) and the conservation of cysteine residues involved in disulphide bonds (67,68) suggests that the two proteins may assume a similar tertiary structure and may share common stereo-epitopes. It has been proposed that this homology may explain some pathological symptoms observed in the Graves' ophthalmopathy (GO) (69), including the lymphocytic infiltration seen in the extra-ocular muscles. To test this hypothesis, we have investigated the possibility of cross-reactivity between antibodies to thyroglobulin and ChE. For this purpose, Igs from patients suffering from GO were interacted with polyclonal antibodies to both proteins in dot blots and protein electrophoretic blots in which the N-terminal part of recombinant human ChE, which displays particularly high homology to Tg, was compared with the conventionally prepared human Tg. In situ studies have further been performed to determine whether patients' Igs would bind to endplate regions of muscle, which are rich in ChE activity, as demonstrated cytochemically.

I.6. Expression of cholinesterase genes in human oocytes approached by in situ hybridization

The involvement of cholinergic mechanisms in oocyte growth and maturation (47,70,71) and sperm-egg interaction (7) has been a subject of contention. Muscarinic ACh receptors were detected in the cell membranes of oocytes of mouse (70), monkey, rabbit and man (72) and Xenopus (73,74), where ACh-induced signal responses regulate the metabolism of phosphoinositides and mobilization of intracellular calcium ions (75). ACh, found in mammalian sperm cells, induces polyspermy in sea-urchin eggs (71), whereas the cholinergic antagonist 3-quinuclidinylbenzilate (QNB) prevents fertilization in mouse (76).

Finally, the ACh-hydrolyzing enzyme, AChE, which terminates ACh responses, was detected in Xenopus oocytes (45,47,77). However, biochemical determinations could not reveal whether AChE is synthesized in the oocyte itself or in surrounding cells (77). This issue is important for human in-vitro fertilization (78), which might involve cholinergic processes. Here we demonstrate pronounced expression of human cholinesterase genes in developing oocytes by in-situ hybridization, supporting the notion that ACh responses in human oocytes may function independently of surrounding follicular cells.

I.7. Could organophosphorous poisons induce the amplification of cholinesterase genes in humans?

Mammalian cell cultures and tumor tissues often achieve resistance to drugs or inhibitors by selection of cells with inheritable amplified genes producing the target protein of such agents (79,80). The ubiquitous enzyme BuChE is strongly inhibited by OP compounds (11) or agricultural insecticides such as parathion (p-nitrophenyl diethyl thionophosphate) (3), to which individuals with "silent" BuChE genes (81) are particularly sensitive. BuChE is expressed early in embryogenesis (82-84), and in situ hybridization using cloned BuChEcDNA revealed BuChEcDNA-positive sites on human chromosomes Nos. 3 and 16 (85) and high levels of BuChEmRNA in developing human oocytes (86). During the course of this research, we found an individual expressing an unusual, defective serum BuChE with a de novo ca 100-fold amplification within a genomic DNA fragment hybridizing with BuChEcDNA and localized on chromosome No. 3. A similar amplification was found in one of his sons, suggesting that it initially occurred very early in embryogenesis, in spermatogenesis or in oogenesis.

I.8. Somatic gene amplification in blood cells may implicate cholinesterases in hemocytopoiesis

In addition to its presence in the membranes of mature erythrocytes, AChE is intensively produced in developing blood cells in vivo (87) and in vitro (88), and its activity serves as an accepted marker for developing mouse megakaryocytes (89). Furthermore, administration of ACh analogues as well as ChE inhibitors has been shown to induce megakaryocytopoiesis and increased platelet counts in the mouse (89), implicating this enzyme in the commitment and development of these hematopoietic cells.

Using the cloned cDNA coding for human BuChE (43) we have localized BuChEcDNA-hybridizing sequences to chromosome sites 3q21-26 and 16q12 (85). It is of importance to emphasize that the chromosome 3q21-26 region includes breakpoints that were repeatedly observed in peripheral blood chromosomes from patients with acute myelodysplastic leukemia (AML, refs. 90,91). These cases all featured enhanced megakaryocytopoiesis, high platelet count and rapid progress of the disease (92). Many recent reports implicate chromosomal breakpoints with molecular changes in the structure of DNA and the induction of malignancies (93). Therefore, the connection between a) abnormal control of megakaryocytopoiesis in AML as well as in mouse bone-marrow cells subjected to ChE inhibition, b) cholinesterase genes' locations

on the long arm of chromosome 3, and c) chromosomal aberrations in the same region in AML appears more than coincidental (see ref. 85 for discussion of this issue).

In order to examine the putative correlation between the human genes coding for ChEs and the regulation of hematopoiesis, or more specifically, megakaryocytopoiesis, we initiated a search for structural changes in the human ACHE and CHE genes from peripheral blood DNA in patients with leukemia, platelet count abnormalities, or both. Our findings demonstrate a significant co-amplification of both the ACHE and CHE genes in peripheral blood cells in patients with leukemia and/or abnormalities in their platelet counts, and strongly suggest an active role for these enzymes in the progress of human hemocytopoiesis.

II. Experimental Observations

II.1. Sequence similarities between human acetylcholinesterase and related proteins

To search for cDNA clones encoding human AChE, oligodeoxynucleotide probes were synthesized according to the amino acid sequences in evolutionarily conserved and divergent peptides from electric fish AChE (63) as compared with human serum BuChE (43,68,94). These synthetic oligodeoxynucleotide probes were used for a comparative screening of cDNA libraries from several human tissue origins.

Previous biochemical analyzes rolvealed that in the brain, the ratio AChE:BuChE is close to 20:1 (25). In contrast, we found the cDNA library from fetal human liver to be relatively rich in BuChEcDNA clones (43). We therefore searched for cDNA clones that would interact with selective oligodeoxynucleotide probes, designed according to AChE-specific peptide sequences in cDNA libraries from fetal brain origin, and particularly from brain basal ganglia that are highly enriched with cholinoceptive cell bodies. Positive clones were then examined for their relative abundance in brain-originated cDNA libraries, as compared with liver. Brain-enriched cDNAs were further tested for their capacity to hybridize with oligodeoxynucleotide probes, previously designed according to the consensus amino acid sequence at the active esteratic site of ChEs (94).

The screening efforts resulted in 25 cDNA clones encoding human AChE sequences extending from the 3' terminus to within an estimated 350 nucleotides from the initiator AUG. DNA sequence analysis followed by computerized alignment of the encoded primary amino acid sequences of human AChE and the previously determined BuChE demonstrated, as expected, that the functional similarity among ChEs reflects genetic relatedness. Final sequence data are being reconfirmed and will be presented in the next report. However, several conclusions may already be derived, as follows.

The active site peptide of human AChE, as deduced from the AChEcDNA clones, revealed 17 of 21 amino acid residues identical to those of either human BuChE or Torpedo AChE (Figure 1). A lower level of similarity (12 of 21 amino acid residues) was observed in comparison with Drosophila AChE (95). Esterase 6 from Drosophila (96) displayed 10 identical residues of these 21, and several serine proteases, 3 or 4 identical residues only (Figure 1). This comparison draws a distinct line between serine proteases and the family of carboxylesterases, and more particularly, the highly conserved ChEs.

The coding region in human AChEcDNA and the inferred amino acid sequence of the human AChE protein were compared with the parallel sequences of human BuChEcDNA (43,94,62) and also with the amino acid sequence of AChEcDNA from Torpedo (63) and of the more evolutionarily remote AChEcDNA from Drosophila (95) (Figure 2). This analysis revealed several peptide regions and DNA sequence domains that are highly conserved in all of the ChEs, particularly at the N-terminal part of the proteins. It further displayed clearly the higher level of divergence between between human and Drosophila AChEs, as opposed to

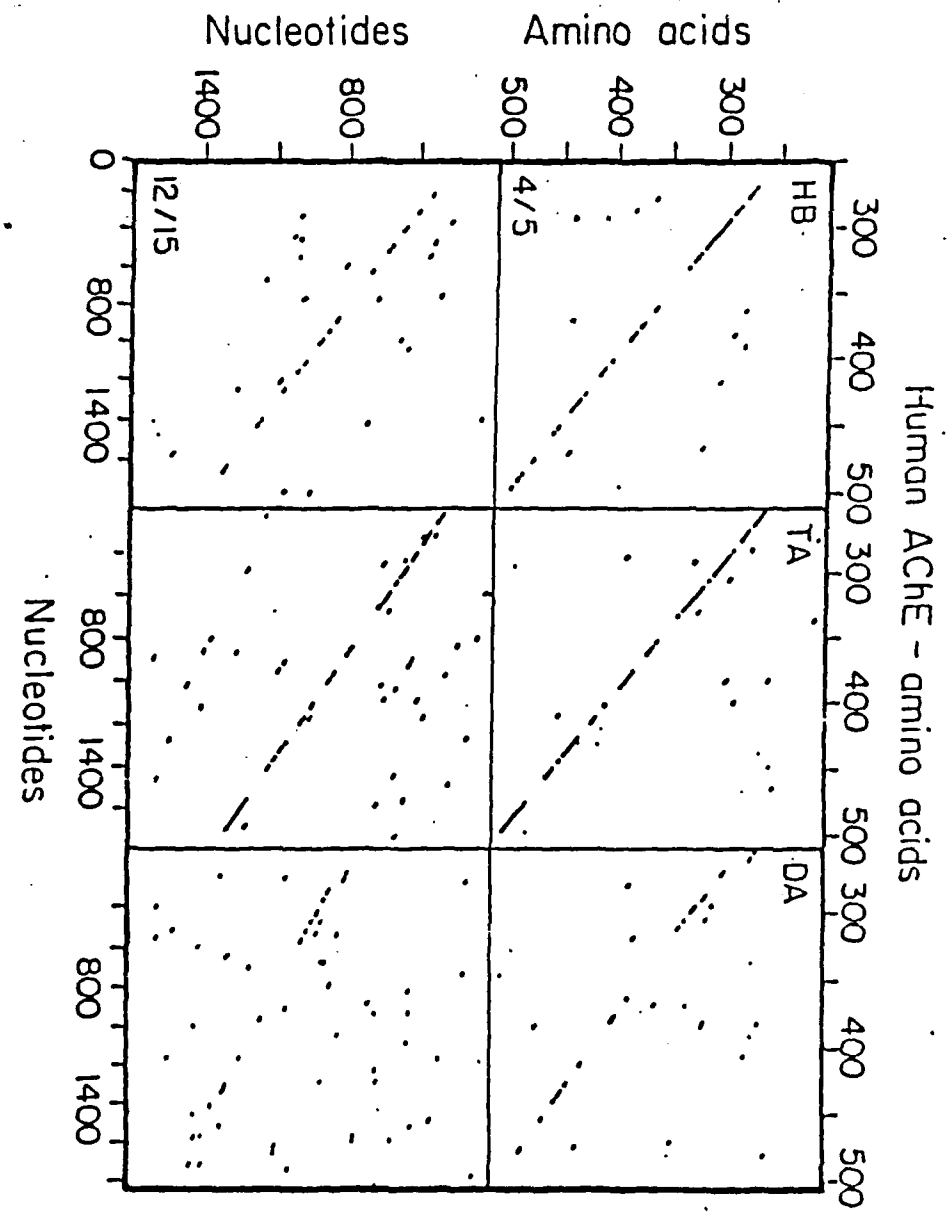
Active Site Homologies

A.	*
Hu. BuChE	NPKSV--TLFGESAGAASVSLHL
Hu. AChE	DPTSV--TLFGESAGAASVGMHL
Tor. AChE	DPKTV--TIFGESAGGASVGMHI
Dros. AChE	NPEWM--TLFGESAGSSSVNAQL

B.	
Dros. Est 6	EPENV--LLVGHSAGGASVHLEM
Pig Elastase	-GNGVRSGCQGDSGGPLV--CQK
Bov. Chymotrypsin	-ASGV-SSCQGDSGGPLV--CQK
Bov. Prothrombin	EGK-RGDACEGDSGGPFVMKSPV
Bov. Factor X	DTQPE-DACQGDSGGPHV--TRF
Hu. Plasminogen	G--T--DSCQGDSGGPLV--CFE
Alpha lytic Protease	IQTNV-CAEPGDSGGSL

C. Active Site Homology of Hu. AChE to:	Estimated Overall Homology of Hu. AChE to:
Hu. BuChE - 85%	Hu. BuChE: 51%
Tor. AChE - 78%	Tor. AChE 56%
Dros. AChE - 52%	Dros. AChE 31%

Figure 1. Comparison of ChE active site region sequences with other serine hydrolases. The asterisk indicates [3H]-DFP-labeled or active site serine. Amino acid sequence data were based on DNA sequencing of human AChEcDNA clones and follow reference nos. 63,94-96 regarding the other active site sequences. Note the considerable difference between the levels of sequence similarities within the ChE family (A) and other serine hydrolases (B). Active site and estimated overall homologies between human AChE and related ChEs are shown in C.



ChE HOMOLOGIES

Figure 2. Amino acid (up) and nucleotide (down) similarities between the coding regions in most of the human AChEcDNA sequence and the parallel regions in cDNAs coding for human BuChE, Torpedo AChE, and Drosophila AChE. Regions of homology were searched for by the dot matrix approach (5, 6). Match values that yielded regions of clear homology and minimal background noise are presented: 12 out of 15 conservative matches for nucleotide sequences and 4 out of 5 conservative matched for amino acid sequences. Nucleotides are numbered in the 5' to 3' direction and amino acids in the N' to C' direction for all sequences.

the extensive similarities between human AChE and BuChE and Torpedo AChE. A higher level of conservation was found for all of these proteins and cDNAs at the amino acid level than at the nucleotide level. This was in complete agreement with previous observations on this gene family and its protein products (5,43). Significant homology was also observed with the DNA and the amino acid sequence of bovine Tg, in corroboration of previous findings (5,63)

To further examine the molecular properties of the human AChE protein encoded by the newly isolated cDNA clones, we subjected it to hydrophobicity analysis (97) (Figure 3). The results of this analysis were compared with parallel analyses of the homologous sequences of human BuChE, Torpedo AChE and Drosophila AChE. The hydrophobicity patterns predicted by this analysis reveal, in all four cases, putatively globular proteins with very short regions of limited hydrophobicity that appear in the same highly conserved positions in the entire family.

In order to search for specific conserved domains in human AChE that could be potentially involved in its hydrolytic activity, the above data were combined with generally accepted concepts of the catalytic functioning of carboxylesterases and of serine proteases. Several serine proteases have recently been subjected to site-directed mutagenesis (see, for example, 98). In corroboration of previous enzymology studies, these recent experiments have demonstrated beyond doubt that three key residues are involved in the charge-relay mechanism of serine proteases, donating protons to a peptide bond which is subsequently hydrolyzed. These include the active site serine, a basic histidine residue and an acidic aspartate residue. In most of the serine proteases, the three key residues appear in the order His-Asp-Ser with an average distance of 43 and 91 residues between the His and the Asp and between the Asp and the Ser, respectively (99). Each of these key residues is embedded in highly conserved peptides, 8-18 amino acids in length. The sequence similarities in the peptides surrounding the reactive site serine 198 are cited in Figure 1. Careful analysis revealed an invariant aspartate at position 170 that is also surrounded by a highly conserved domain, as expected from residues playing important roles in hydrolytic activity. In spite of the non-conserved distance between this Asp 170 and Ser 198, these two residues appear to be very good candidates for the putative key functions in the charge-relay system.

The high pH dependence of the catalytic activity of ChEs (100) and their sensitivity to chemical agents that modify imidazole groups (101) suggest that a histidine residue is also involved in the charge-relay mechanism of ChEs. However, there is no conserved histidine on the amino-terminal site of the reactive serine. On the other hand, a highly conserved peptide including an arginine residue can be found around position 147. Arginine replaces histidine in the charge-relay system of phospho-diesterase ST (102), suggesting this residue as a substitute for the conservative His.

An alternative possibility suggests that histidine residues in other positions take part in the charge-relay system of ChEs. Indeed, highly conserved peptides that include histidine residues may be found

COMPARATIVE ChE HYDROPHOBICITY PROFILES

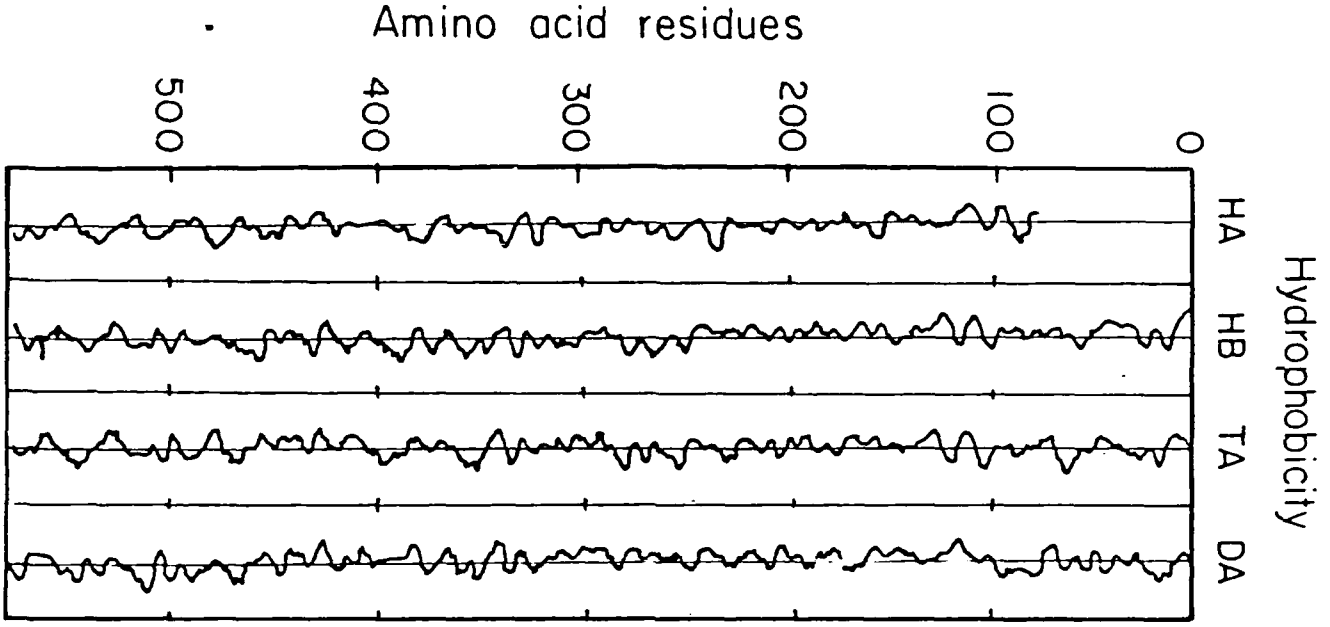


Figure 3. Comparative hydrophobicity patterns of members of the ChE family. The predictions of the hydropnobic and hydrophilic regions of human AChE and BuChE, Torpedo AChE, and Drosophila AChE are presented 5, 6). The dotted vertical baseline in each box represents a hydrophobicity value of 0; increasing hydrophylicity is indicated in the right-hand direction and increased hydrophobicity in the left-hand direction.

at positions 423 and 438. Both were suggested by Sikorav et al. (103) to take part in the charge-relay mechanism. According to this proposal, the basic histidine residue would therefore be located on the carboxy-side of the reactive serine. An example for reversed positions of the Asp and His residues relative to the Ser may be found in another serine protease, subtilisin (104).

Site-directed mutagenesis studies will be required to distinguish between the above-discussed possibilities for putative residues in human AChE. However, in all cases, ChEs are indicated to have a different charge-relay system from that of serine proteases, differing in the identity of the basic residue, in its distance from the reactive Ser or in its relative position on the primary sequence. The possible combinations for the charge-relay key residues of ChEs, including the peptide similarities, are presented in Figure 4.

II.2. Isolation and characterization of BuChEcDNA from adult liver cDNA library

The results of our cloning studies clearly demonstrated that within the fetal brain and liver, AChE and BuChE is produced from two distinct mRNA transcripts. However, it could not answer the question whether either AChE or BuChE is produced from different transcripts in fetal as compared with adult tissues. To approach this issue, cDNA screening was performed in parallel in cDNA libraries from fetal and adult livers. The first probes employed were those coding for BuChE. Thus we have screened a cDNA library from adult liver origin and constructed in a lambda GT10 vector in order to isolate cDNA clones coding for BuChE. Two main probes were used to detect the presence of the right cDNA insert by hybridization:

1. The full-length BuChEcDNA clone previously isolated from human fetal brain and liver libraries (43), which was found to code for the catalytic subunit of BuChE as it appears in the adult serum (68);
2. A cDNA clone coding for the 200 first N-terminal amino acid of this protein (94).

It was known that the coding region could not be subject to large variations, since the protein inferred from it remains the same in enbryonic and adult tissues. However, we searched for 3'-variant cDNAs coding for BuChE, in analogy to those for AChE in Torpedo (103). The 5'-partial probe should in principle pick these clones with no difficulty. The results of the screening experiment demonstrated that BuChEcDNA inserts were much less abundant in the adult liver library than in the fetal liver one. Only three clones out of $5 \cdot 10^5$ were positive after the third screen with the BuChEcDNA probe, as compared with 40 in a parallel screen of the fetal liver library. One of these inserts has been purified and then subcloned into the sequencing single-stranded phage M13.

Nucleotide sequencing of the full-length adult liver BuChEcDNA has been performed using the dideoxy Sanger method as previously described (43, 105). The nucleotide sequence of the adult liver BuChEcDNA was identical to the fetal one in all respects, clearly

Putative residue combinations for the charge-relay system of the ChE catalytic sites.

A-C. Key residues combinations

A. Arg147 - Asp170 - Ser198 (23 - 28)
B. His423 - Asp170 - Ser198 (253 - 28)
C. His438 - Asp170 - Ser198 (268 - 28)

D. Sequence Similarities

	Arg147 region	Asp170 region	His423 region	His438 region
Hu. AChE	yRvgafgflal	nvgllDqrlal	Hrastlswmgv	pHgveieftfg
Hu. BuChE	yRvgalgflal	nmglfDqqlal	Hrssklpwmgv	mHgveiefvfg
Tor. AChE	yRvgafgflal	nvgllDqrmal	Hrasnlvwmgv	iHgveiefvfg
Dros. AChE	yRvgafgflhl	nvglwDqalai	Hrtstslwmgv	lHgdeieyffg

Figure 4. A-C. Possible combinations for the key residues in the charge-relay system of human cholinesterases are presented. Residues are numbered according to their appearance in the mature human BuChE protein. The numbers of residues between the 1st and 2nd pairs of amino acids are marked in parentheses for each combination. D. Sequences were aligned as previously detailed (5,43) for human AChE (Hu. AChE), human BuChE (Hu. BuChE), Torpedo AChE (Tor. AChE) and Drosophila AChE (Dros. AChE). The position of each putative catalytic site residue within the surrounding 11 amino acid sequence is shown above each region. Residue numbering begins with 1 as the first amino acid of the mature human BuChE protein (68), since the human AChE sequence is deduced from cDNA data only. The putative catalytic residues are shown in upper case letters.

demonstrating that it was transcribed from the same gene by a similar mechanism of post-transcriptional processing (Figure 5).

II.3. In ovo translation of synthetic BuChEmRNA in microinjected Xenopus oocytes

Our early attempts to express the human BuChE polypeptide in a bacterial expression system (in collaboration with J. Hartman and M. Gorecki, Biotechnology General, Nes-Ziona) resulted in the production of a totally inactive, although immunochemically positive protein (unpublished). We therefore proceeded to express human BuChEcDNA in Xenopus laevis oocytes, known to be capable of translating native human ChEmRNAs (45)

a. Oocytes injected with synthetic BuChEmRNA produce active butyrylcholinesterase

The synthetic mRNA we injected was transcribed from a cDNA construct containing the binding site for the SP6 salmonella phage RNA polymerase followed by the cDNA sequence (43) encoding an amino acid sequence which is identical to that of human serum BuChE (68) (Figure 6). This enzyme may be distinguished from the other major cholinesterase, AChE, by its substrate preference and sensitivity to selective inhibitors. In our first experiments we therefore assessed the possibility that the primary amino acid sequence is sufficient to confer ligand binding specificity.

The enzyme produced by the oocyte in response to injection of SP6BuChEmRNA demonstrated a clear preference for butyrylthiocholine (BuSCh) over acetylthiocholine in activity assays. Enzymatic activity averaged 8.2 +/- 1.6 nmoles BuSCh hydrolyzed/hr/oocyte - 2 orders of magnitude higher than the activity achieved with total poly(A)$^+$ RNA from embryonic brain (46) (Figure 7). By comparison, acetylthiocholine hydrolysis ranged 3-4 times lower, averaging 2.2 +/- 0.7 nmole/hr/oocyte. An apparent Km of 2 x 10^{-3} M BuSCh was determined for the secreted, cytoplasmic, and membrane-bound fractions of the enzyme (Table I). This value corresponds to values calculated for several control human serums in our laboratory.

The total BuChE activity measured in injected oocytes was distributed as follows: 4%/-1% in the medium, 38%/-7% low salt soluble and 58%/-7% extracted. The oocyte enzyme exhibited characteristic inhibition by the BuChE-specific OP inhibitor tetra-isopropylpyrophosphoramide (iso-OMPA) while resisting inhibition by the AChE-specific quaternary inhibitor 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51).

The IC_{50} values 2-3 x 10^{-6} M iso-OMPA calculated for the secreted and cytoplasmic pools of the enzyme were essentially identical to the value determined in parallel for the human serum enzyme, while the IC_{50} value 1 x 10^{-5} M measured for the detergent-extracted enzyme indicates a limited but significant decrease in BuChE affinity for this inhibitor in the presence of detergent (Figure 8). All three fractions displayed a 10-20% inhibition by high concentration (10^{-4}M) of BW284C51.

ADULT LIVER BuChEcDNA

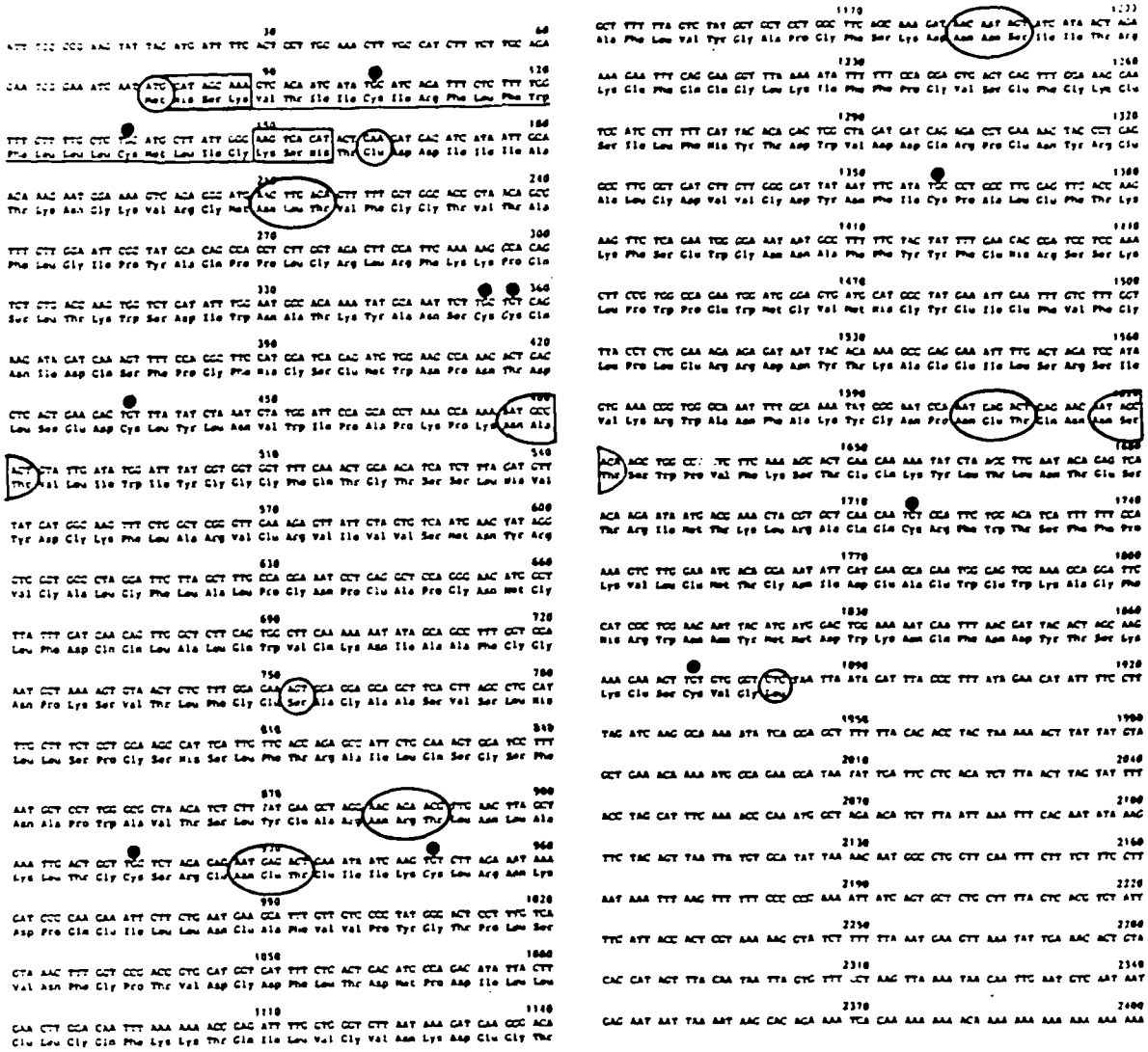


Figure 5. The nucleotide sequence of full-length BuChEcDNA isolated from an adult human liver cDNA library and its encoded amino acid sequence are shown. Circled are the initiator methionine, the terminal amino acid of the putative signal peptide, the active-site serine and the terminal amino acid. Indicated by closed circles are cysteine residues. Putative glycosylation sites are marked with an oval. The putative signal peptide is underlined.

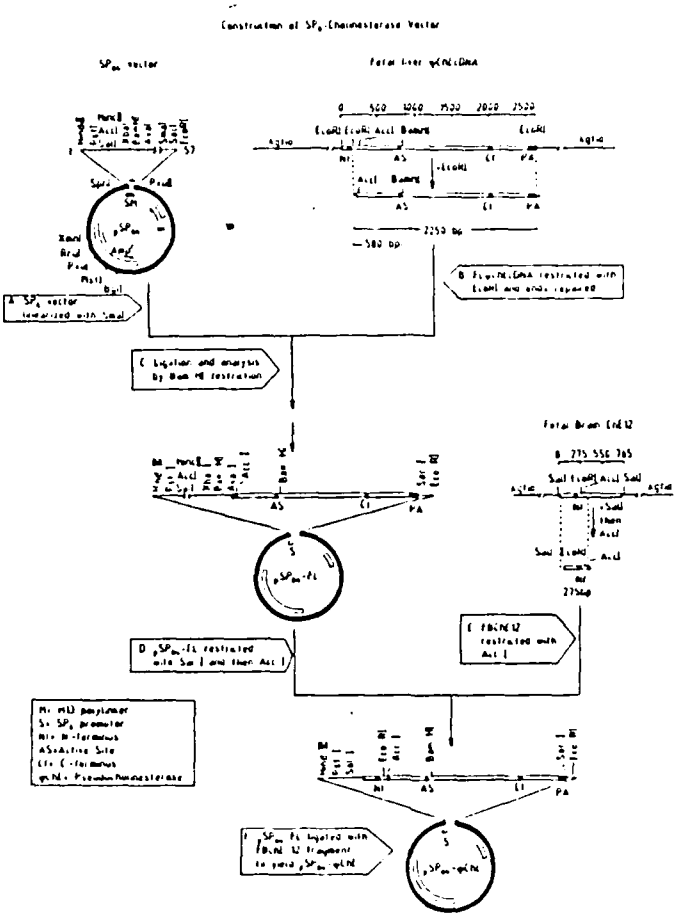


Figure 6. The fetal liver BuChEcDNA Lambda gt10 clone, containing an insert of 2450 nucleotides in length (47) was digested by EcoRI producing Lambda gt10 "arms" and two fragments of the BuChEcDNA insert: a 2250 base-pair fragment (FLBuChEcDNA) which contained most of the cDNA sequence, and a 190 nucleotide fragment from the 5'-terminal domain coding for the signal sequence and the N-terminal part of the BuChE protein. The FLBuChEcDNA was isolated by preparative gel electrophoresis and electroelution. The cohesive ends produced by EcoRI restriction were repaired using the Klenow subunit of DNA polymerase and the resultant FLBuChEcDNA fragment was blunt-end ligated into the SmaI site of the linearized pSP64 vector. Restriction of plasmid DNA by BamHI produced a fragment of 580 base pairs demonstrating that the insert was introduced in the correct orientation. The fetal brain FBChE12 clone (94) was employed to prepare the region coding for the N-terminal domain of BuChE. Insert DNA was first excised with SalI and then restricted with AccI, to yield a 275 base-pair fragment with the appropriate enzyme sites for insertion into the restricted pSP64-FL constructs. The SalI-AccI fragment of FBChE12 was purified and ligated into the SalI-AccI cut pSP64-ChE plasmid, thus constructing the pSP64-ChE plasmid containing the complete ChEcDNA introduced in the correct orientation for <u>in vitro</u> transcription.

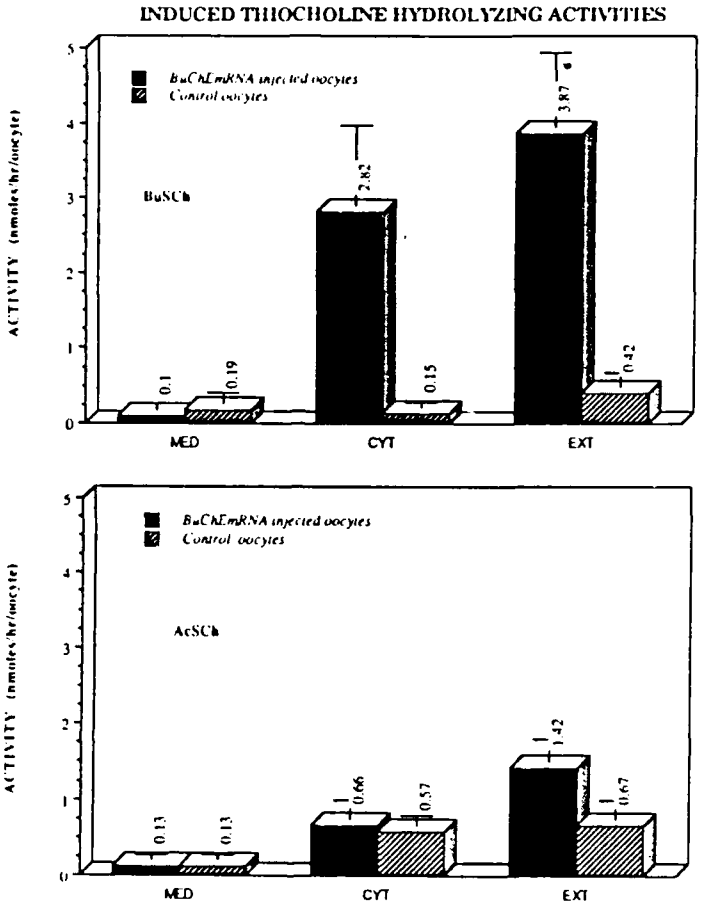


Figure 7. Values represent the average net BuSCh (up) and AcSCh (down) hydrolyzing activities (expressed in nmoles/hr/oocyte) +/- SEM (bar) for 5 independent experiments in subcellular fractions from oocytes injected with synthetic BuChEmRNA. A - incubation medium, B - low-salt-soluble fraction, C - high salt, detergent extracted fraction. Spontaneous substrate hydrolysis and control oocyte activities were subtracted for each experiment. Average control activities +/- SEM for the same 5 experiments are shown in parallel. Subcellular fractionations and activity assays were carried out as detailed in Experimental Procedures.

SUMMARY OF Km CALCULATIONS

	MEDIUM			CYTOPLASM			EXTRACT		
	I	II	III	I	II	III	I	II	III
AcSCh									
LWB/HAND	•••	•••	•••	4.40E-03	•••	•••	2.20E-03	1.80E-03	•••
LWB / L.R.	•••	•••	•••	9.40E-04	•••	•••	2.00E-03	1.70E-03	•••
LWB/LOTUS	•••	•••	1.00E-03	•••	•••	2.50E-03	•••	•••	1.25E-03
EISEN-CORNISH	•••	•••	•••	•••	•••	•••	•••	1.50E-03	•••
KMVM	•••	•••	2.50E-03	7.00E-05	•••	2.20E-03	1.60E-03	•••	2.80E-03
HYPERBOL	•••	•••	1.70E-03	9.20E-03	•••	3.40E-03	1.40E-03	4.40E-03	3.00E-03
BuSCh									
LWB/HAND	•••	•••	•••	6.30E-04	•••	•••	2.20E-03	5.00E-03	•••
LWB / L.R.	•••	•••	•••	2.00E-03	1.00E-03	•••	1.50E-03	6.20E-03	•••
LWB/LOTUS	•••	•••	5.00E-03	•••	•••	2.50E-02	•••	•••	5.00E-03
EISEN-CORNISH	•••	•••	•••	•••	1.50E-03	•••	•••	•••	•••
KMVM	•••	•••	2.00E-03	3.20E-03	1.00E-02	2.00E-02	2.90E-03	2.70E-03	2.70E-03
HYPERBOL	•••	•••	2.50E-02	2.50E-03	•••	2.00E-02	6.70E-03	1.20E-03	1.70E-03

Km DETERMINATIONS OF OOCYTE PRODUCED BuChEs

Table I - Summary of K_m determination results. Enzymatic activity was measured as a function of substrate concentration in three subcellular fractions from three independent microinjection experiments (I, II, III). The data from each experiment were subjected to K_m analysis using a variety of methods. The results are summarized in the table: LWB - Lineweaver-Burke calculated by "hand" ie. best-fit line by eye; by "LR" ie. linear regression analysis; by "Lotus" ie. a combination of graphical and mathematical analyses performed using the Lotus 1-2-3 computer program. Eisen-Cornish - A graphical method perfomed using "pencil, paper, and ruler." KMVM - A non-linear regression analysis performed on our Olivetti M24 personal computer. HYPERBOL - a hyperbolic curve fitting program also executed on the Olivetti P.C. Results were weighted and averaged by "eye," (not all methods were given equal consideration) and an apparent K_m of 2-3 x 10^{-3} M BuSCh was estimated for all three subcellular fractions. Similar results were obtained with native human serum from several apparently healthy adult subjects (not shown).

INHIBITION OF THE OOCYTE-SYNTHESIZED ENZYME BY THE BuChE-SPECIFIC ORGANOPHOSPHOROUS INHIBITOR iso-OMPA

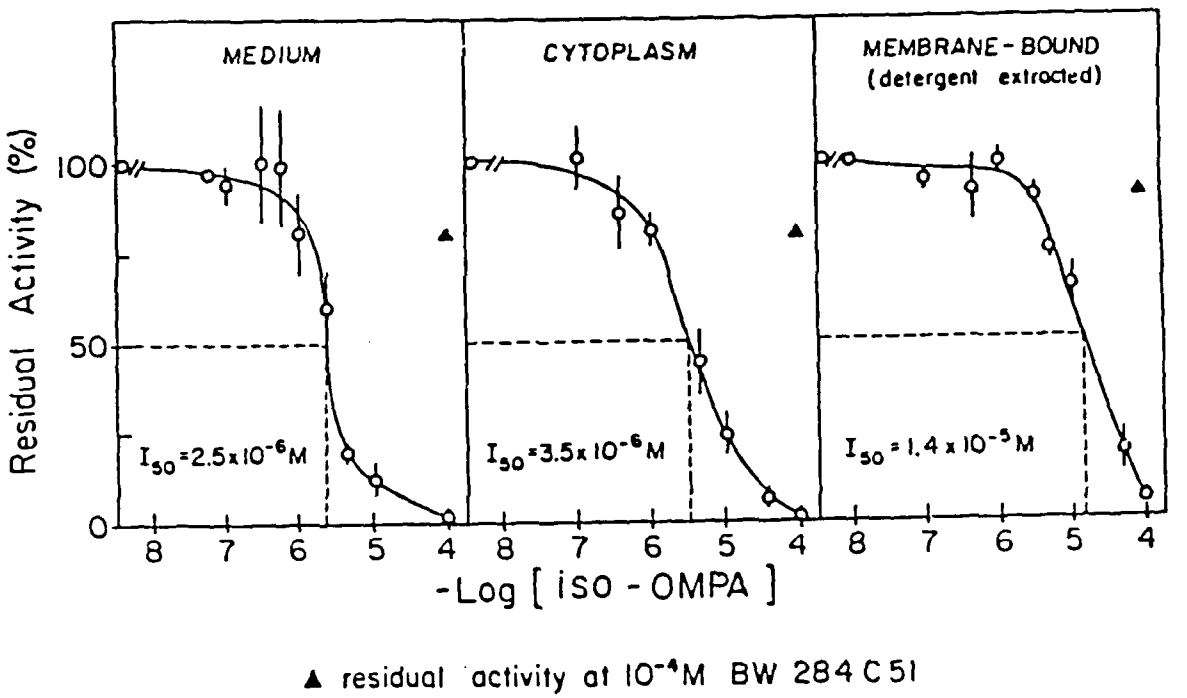


Figure 8. Oocytes were microinjected with synthetic BuChEmRNA and incubated 18 hrs at 18°C. Subcellular fractions ("Medium," "Cytoplasm," and "Membrane-bound") were prepared as detailed under Methods. 5 ul samples were assayed for BuSCh hydrolyzing activity in the presence of varying concentrations of inhibitor. Results represent average values +/- standard evaluation of the mean for 3 independent microinjection experiments. Note the minimal inhibition effected by the AChE-specific inhibitor BW284C51. The IC_{50} determined for human serum BuChE under identical assay conditions was 1.3 X 10^{-6} M iso-OMPA (not shown).

b. Synthesized butyrylcholinesterase dimers assemble into complex multimeric forms following co-injection with tissue RNAs

Linear sucrose gradient ultracentrifugation was employed to examine the nature of subunit assembly in clone-produced BuChE. When injected with synthetic BuChEmRNA alone, all three subcellular oocyte fractions contained distinct BuChE activity displaying a sedimentation coefficient of 5-7S and corresponding to the globular dimeric enzyme form (Figure 9a). In contrast, native serum BuChE exists primarily as globular tetramers (10). Supplemental muscle poly(A)$^+$ RNA induced a complete array of BuChE molecular forms in the membrane-associated fraction of the oocytes, including a heavy 16S peak characteristic of the neuromuscular "tailed" form of the enzyme (24), which represents a significant fraction of human fetal muscle BuChE (Figure 9b, up). Co-injection of fetal brain poly(A)$^+$mRNA with the synthetic BuChEmRNA induced a considerable peak of membrane-associated BuChE activity sedimenting as 12S globular tetramers the primary BuChE form found in human fetal brain (25) (Figure 9b, down).

c. Clone-produced butyrylcholinesterase associates with the oocyte surface.

In human brain and muscle, BuChE associates with the extracellular surface from which it can be detached by salt and detergent (25,107). To examine the mode of association of the clone-produced enzyme with the external surface of the injected oocytes, an immunohistochemical approach was taken. Frozen sections of BuChEmRNA-injected oocytes were incubated with antibodies to Torpedo AChE, known to cross-react with human BuChE (108) and then with fluorescein-conjugated second antibody. Characteristic green signals on the oocyte surface could be easily distinguished from the yellow autofluorescence emitted from the internal oocyte yolk vesicles (109) (Figure 10a). Clone-produced BuChE accumulations appeared on the surface of the oocytes in the form of either small, round "clusters," about 5 um in diameter, or elongated "patches," 20 um in diameter (Figure 10b-e). Both types of structures were relatively concentrated at the animal as opposed to vegetal pole, and could be identified within 30 minutes after microinjection. Signal intensity increased with time up to 2.5 hrs after injection, at which point maximum intensities were achieved (Figure 11). In contrast, muscle poly(A)$^+$ RNA, which contains about 0.001% of BuChEmRNA (H. Soreq, unpublished observations), created very weak surface-associated signals. Blockage of glycosylation by tunicamycin induced the accumulation of fluorescent signals around intracellular vesicles (Figure 12).

d. Co-injection with tissue mRNAs intensifies surface-associated butyrylcholinesterase signals

Co-injection of brain and muscle mRNA with the synthetic BuChEmRNA resulted in 2.4- and 3.6-fold increases, respectively, in the total surface area occupied by patches and clusters while maintaining the 2-3-fold disproportionate distribution at the animal over vegetal pole (Table II). In both cases, the relative intensity of fluorescent staining was also increased, although the enhancement

OOCYTE BuChE MOLECULAR FORMS

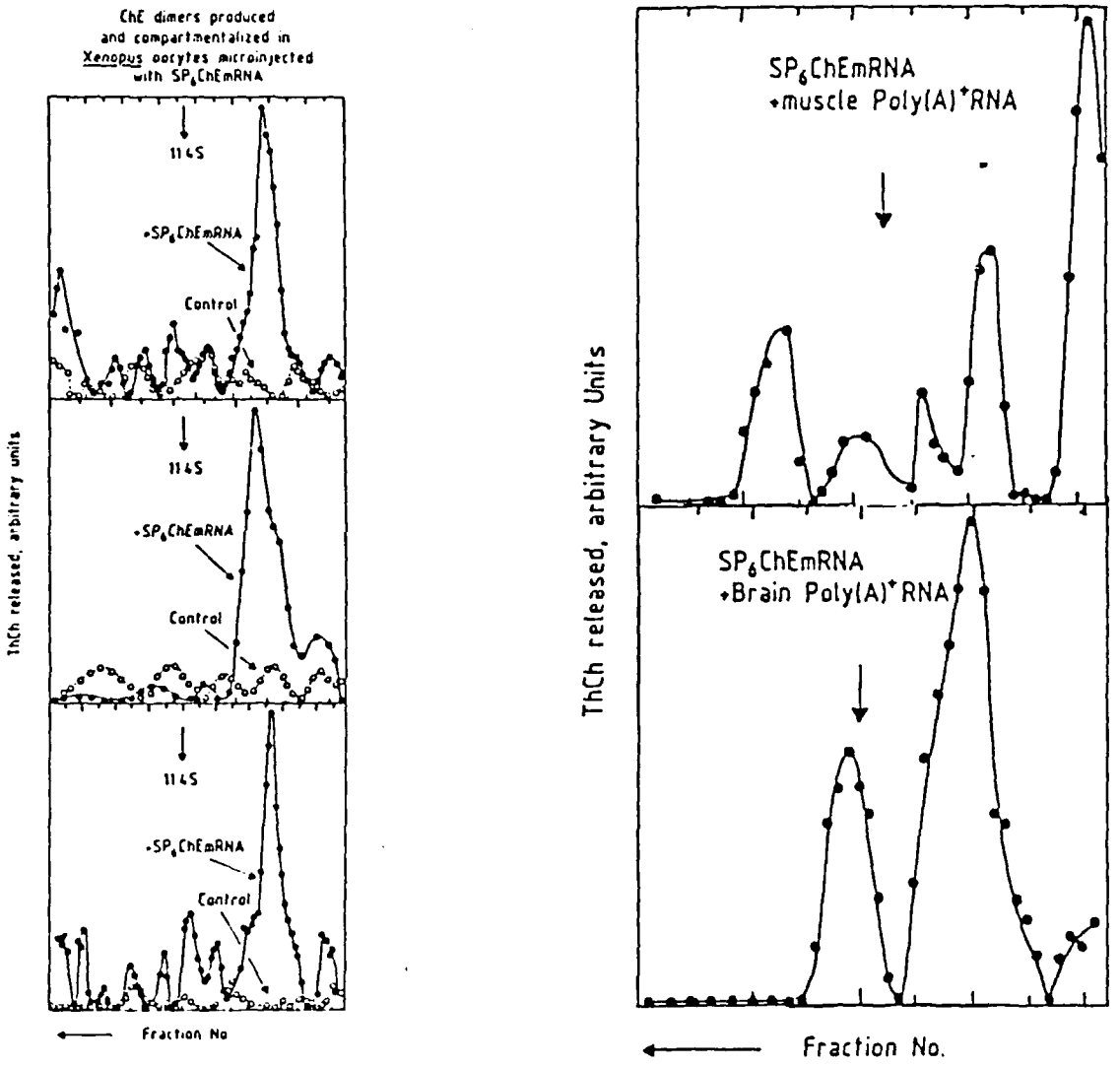


Figure 9. A. Sucrose gradient fractions from 5-10 injected (-●-) and non-injected (-○-) oocytes were assayed for BuSCh hydrolyzing activity and A_{405} vs. fraction number was plotted after subtraction of background. Arrow indicates position of bovine catalase (11.4S) marker. Fraction 0 is bottom of tube. Primarily dimeric forms (approx. 5-7S) can be clearly identified in all 3 subcellular fractions: low-salt-soluble (top), detergent-extractable (middle) and incubation medium (bottom). Small "shoulders" may be indicated in the 3-4S region characteristic of monomeric ChE. One out of three experiments is presented; yields were about 50% of loaded activities. Incubation, detergent extraction, and sucrose gradient analysis were performed with BuSCh as described in Experimental Procedures. B. Gradient analysis of oocytes injected with 5.0 ng of synthetic BuChEmRNA and 25 ng of unfractionated poly(A)$^+$ RNA from muscle or brain. Note that co-injection with tissue-specific poly(A)$^+$ RNA's induced the biogenesis of high molecular weight forms of detergent-extractable ChE. Fetal brain-derived mRNA (down) induced the formation of tetrameric BuChE(12S), while muscle-derived mRNA (up) generated an array of oligomeric forms.

IMMUNOCYTOCHEMICAL ANALYSIS OF SURFACE-ASSOCIATED OOCYTE-PRODUCED ENZYME

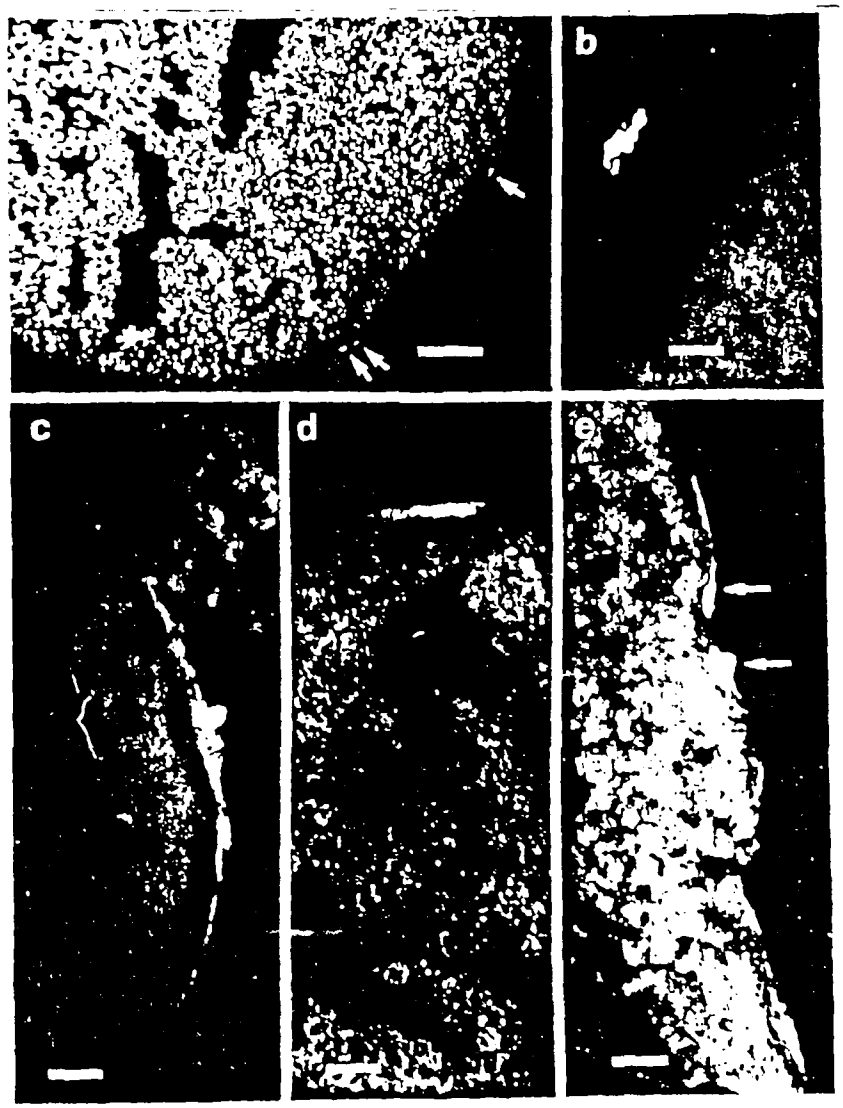


Figure 10. Oocytes were injected with synthetic BuChEmRNA, incubated overnight (A) or for the indicated time (B) at 18°C, frozen, and sectioned as detailed in Methods. Oocyte sections were incubated with cross-reactive polyclonal anti-Torpedo AChE antibodies followed by incubation with rhodamine- or fluorescein-conjugated 2nd antibody and viewed by fluorescence microscopy. a. Few small ChE accumulations on the oocyte periphery (arrow). Scale bar = 100 um. b,c,d,e. Close-up view of some ChE "patches" (b,d) and "clusters" (c,e). Immunofluorescent signals seem to be localized on the extreme external surface of the oocyte. (d) and (e) represent the animal pole of the oocyte. Scale bar = 20 um.

KINETICS OF NASCENT CHE ASSOCIATION WITH OOCYTE SURFACE

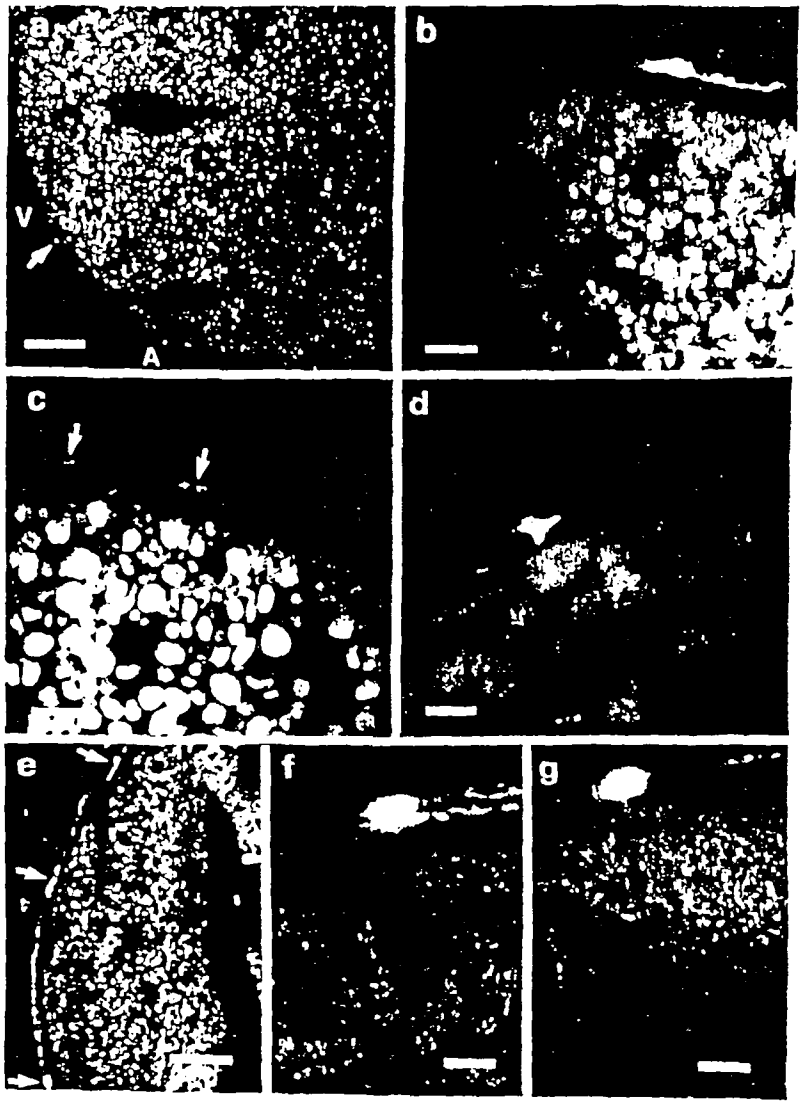


Figure 11. a,b. About 30 minutes post-injection. a: Very rare and small agregates of ChE (arrow) on the oocyte periphery. Animal pole (A), Vegetal pole (V). Scale bar = 100 um. b: Close-up of membrane-associated BuChE accumulation at the Animal pole. Scale bar = 20um. c,d. 45 minutes post-injection. ChE accumulations are very similar to those observed at injection time, but more frequent. c: Vegetal pole. d: Animal pole. Scale bar = 20 um. e,f,g. 2.5 hr post-injection. e: Fluoresecent ChE clusters are bigger (arrow) , more frequent and more intense than those seen at the previous injection time. Animal pole. Scale bar = 100 um. f,g: Close-up view of ChE clusters on the Animal pole. Scale bar = 20um.

SYNTHETIC BuChEmRNA AND GLYCOSYLATION ARE NECESSARY REQUIREMENTS FOR THE DETECTION OF NASCENT ChE MOLECULES AT THE OOCYTE SURFACE

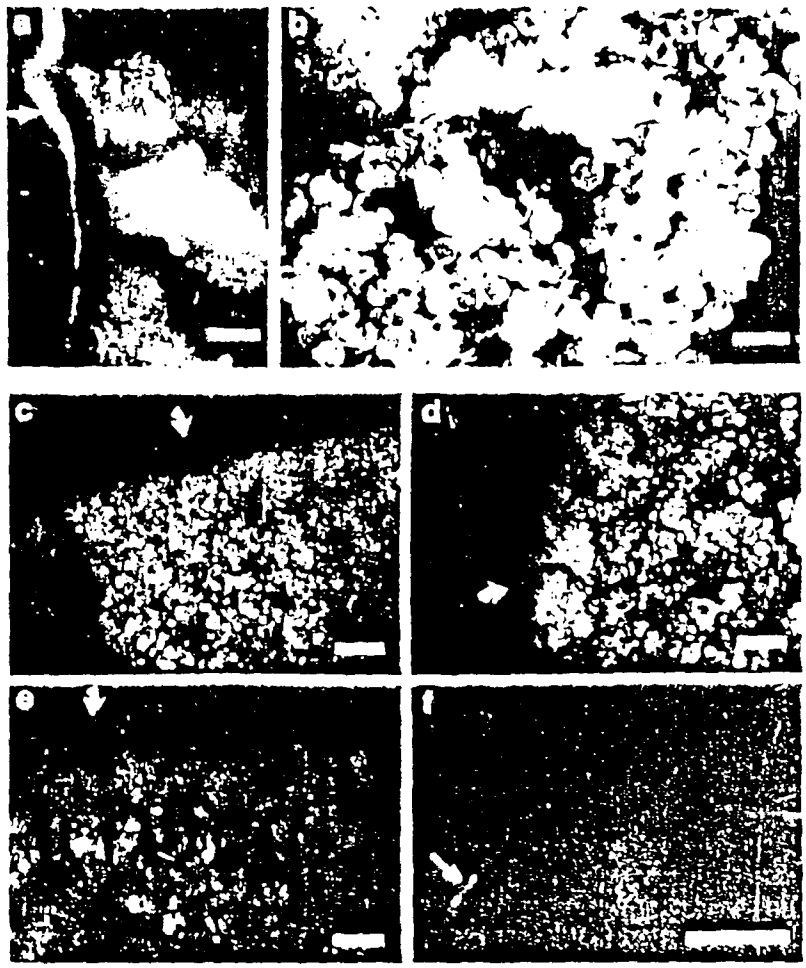


Figure 12. a,b: Oocytes injected and incubated with 10uM Tunicamycin 24 hrs prior to BuChEmRNA injection. Tunicamycin seems to interfere with normal transport of ChE to the oocyte external surface. Scale bar = 20um. a: Diffuse external ChE accumulation (arrow). b: Cytoplasmic ChE accumulation closely associated to yolk vesicles (arrow). Various control experiments. c: BuChEmRNA-injected oocytes treated with rhodamine-conjugated 2nd antibody alone (no anti-ChE). Only the non-specific autofluorescence could be observed. d: Oocytes injected with BuChEmRNA + brain poly(A)$^+$RNA and treated with phycoprobe-conjugated 2nd antibody only (i.e. no immunoreaction.) e: Oocytes injected with Barth medium and treated with anti-AChE 1st antibody and with phycoprobe-conjugated 2nd antibody. Note the absence of immunoreaction. f: Oocytes injected with fetal muscle poly(A)$^+$RNA alone and treated with anti-ChE 1st antibody and with rhodamine-conjugated 2nd antibody. Note the few faint ChE clusters in the oocyte periphery (arrow).

obtained in oocytes co-injected with muscle mRNA was significantly more dramatic (Figure 13). Particularly high accumulations of immunofluorescing structures were detected in oocyte sections which included the injection site.

Electron microscopic analysis using second antibodies coupled to 5nm gold beads confirmed the qualitative differences between the surface-associated BuChE accumulations in oocytes co-injected with brain or muscle mRNA (Figure 14,15). The high sensitivity of analysis at this level further revealed that these BuChE deposits were not primarily associated with the plasma membrane of the oocyte itself, but in fact, were mostly linked closely with the external layer of the extracellular material surrounding the oocytes and their follicle cells. Some gold particles could also be detected at the level of the oocyte microvilli and follicle cells, perhaps caught en route to the cell surface. In tunicamycin-injected cells, immunogold beads were concentrated around intracellular vesicles.

II.4. Cross-homologies and structural differences between cholinesterases revealed by elicitation of antibodies against bacterially produced butyrylcholinesterase polypeptides

In order to search for similarities and differences between various ChEs at the level of the naked polypeptide, the amino acid sequence of human butyrylcholinesterase, as deduced from cDNA sequence information (43), was subjected to computerized analysis by the Chou-Fasman prediction (110). Measures of alpha-helix and beta-sheet values (111,112), "best guess" predictions of immunogenicity (113) and hydropathic characteristics (114) were combined to examine the expected immunogenicity of specific regions within the polypeptide sequence. Using this analysis, the N-terminal 200 amino acids of BuChE were found to be particularly low in immunogenicity. Since this is also the part of BuChE which shows greatest homologies to other ChEs (5), we selected this polypeptide as an antigen. To prepare this protein in a naked form, the FBChE12 insert (43,94) transcribed from BuChEmRNA was used. This insert consists of a fully open reading frame and codes for the signal peptide and the N-terminal 200 amino acids of human BuChE (5). It was subcloned into the pEX bacterial expression vector (115) and ligated to the 3'-end of the gene encoding beta-galactosidase in the pEX plasmid.

a. <u>Expression of a partial BuChEcDNA clone in a bacterial plasmid induces the production of polypeptides that are immunoreactive with various cholinesterase antibodies.</u>

In the absence of insert, shift of bacteria transformed with pEX plasmids to 42°C induces synthesis of beta-galactosidase (115). In contrast, induction of beta-galactosidase expression in bacteria transformed with the pEX$_3$FBChE12 construct resulted in the production of a fusion protein of ca. 125 kd, which was mostly proteolyzed in the bacteria to yield a series of proteolytic products in the range of 35,000-75,000 daltons (Figure 16a). (It should be noted that fusion proteins produced from cDNA inserts of ca. 400 nucleotides or more in pEX vectors tend to be proteolyzed in the bacteria (115)). Also,

BuChE ASSOCIATION WITH OOCYTE SURFACE

injected:[1]	BuChEmRNA alone		BuChEmRNA + Brain mRNA		BuChEmRNA + muscle mRNA	
Hemisphere[2]:	Animal	Vegetal	Animal	Vegetal	Animal	Vegetal
Clusters[3]						
No.	3.5±0.5	3±1	7±1	4.5±0.5	25±2	13±1
(um^2)surface	280	240	560	360	2000	1040
x-fold surface	1.00	1.00	2.00	1.50	7.14	4.33
Patches						
No.	9.5±1.5	6.5±1.5	26.5±1.5	9.5±0.5	34±2	18±1
(um^2)surface	4750	2750	13250	4750	17000	9000
x-fold surface	1.00	1.00	2.79	1.00	3.58	3.27
Total[4]						
(um^2)surface	5030	2990	13810	5110	19000	10040
x-fold	1.00	1.00	2.75	1.71	3.78	3.36

Table II 1. A complete series of 10 um-thick sections from 2-1.2 mm diameter, stage 6 oocytes (Dumont, 1972) microinjected with synthetic SP6BuChEmRNA alone or combinations of this RNA with fetal muscle or brain poly(A)$^+$ RNA, was immunoreacted with pre-adsorbed anti-AChE antibodies and quantitatively analyzed by fluorescence microsopy as described under Methods. RNA was injected at the midline dividing the animal and vegetal poles. Biochemical analyses of the induced BuChE activities in the subcellular fractions of the oocytes used for this analysis were within the average level of induction (see results). 2. Oocyte sections were visually identified as animal or vegetal pole by detection of pigment vesicles at the animal hemisphere. Numbers reflect the total count from all of the sections representing each hemisphere. 3. Positive immunofluorescence signals of 1-10 um diameter were defined as "clusters" with the average surface area of one cluster being estimated as 80 sq. micron. Fluorescent signals of 10-25 um in diameter were defined as "patches" with the average surface area of a single patch being 500 sq. micron. 4. The Total number of clusters and patches were recorded so that signals appearing in 2 adjacent sections were only counted once.

FLUORESCENCE MICROSCOPY ANALYSIS OF FROZEN SECTIONS FROM OOCYTES CO-INJECTED WITH SYNTHETIC BuChEmRNA AND NATIVE POLY(A)$^+$ RNAs.

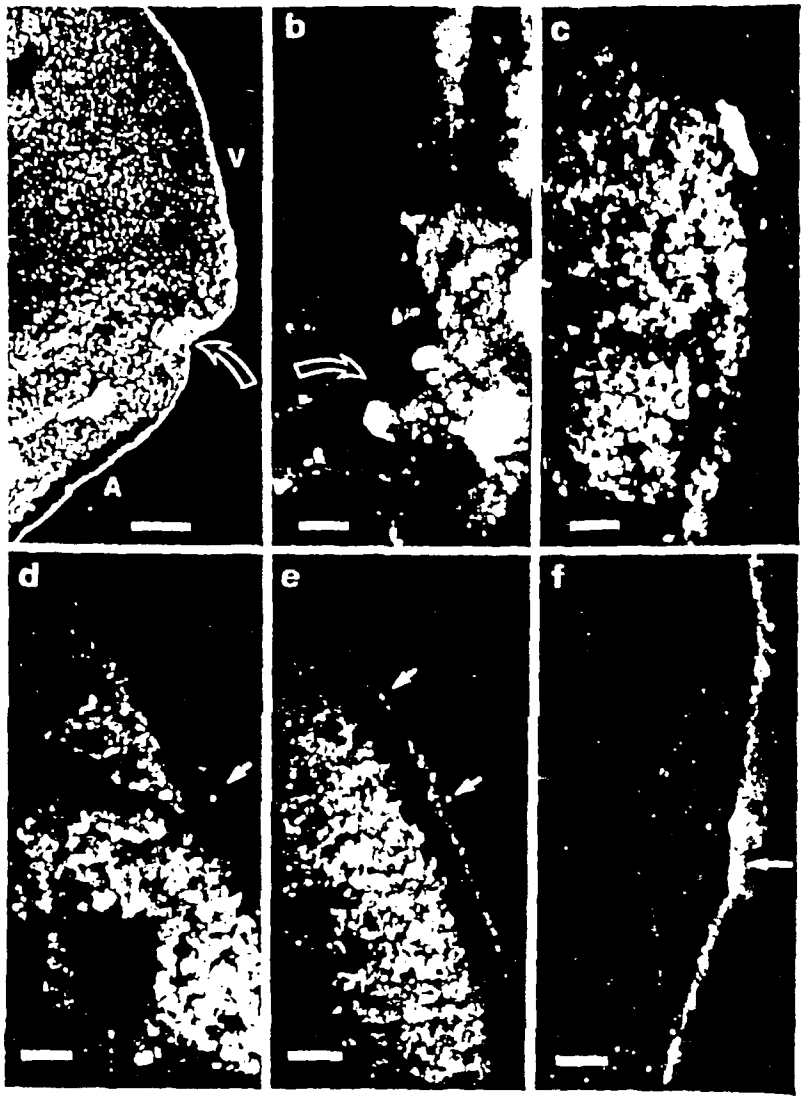


Figure 13 .a,b,c. Oocytes co-injected with synthetic BuChEmRNA and muscle poly(A)$^+$ RNA. a: The injection site (open arrow) is clearly visible between the Animal pole (A) and the Vegetal pole (V). Scale bar = 100 um. b: Close-up view of the injection site (empty arrow). Note the strong ChE immunoreactivity. Scale bar = 20 um. c: A big ChE patch in the most peripheral part of the oocyte. Scale bar = 20 um. d,e,f. Oocytes co-injected with BuChEmRNA and brain poly(A)$^+$ RNA. The ChE activity appears as very small clusters or as diffuse sites also in the internal part of the oocyte membrane (arrow). Scale bar = 20 um.

IMMUNOGOLD ELECTRON MICROSCOPY OF SECTIONS FROM CO-INJECTED OOCYTES.

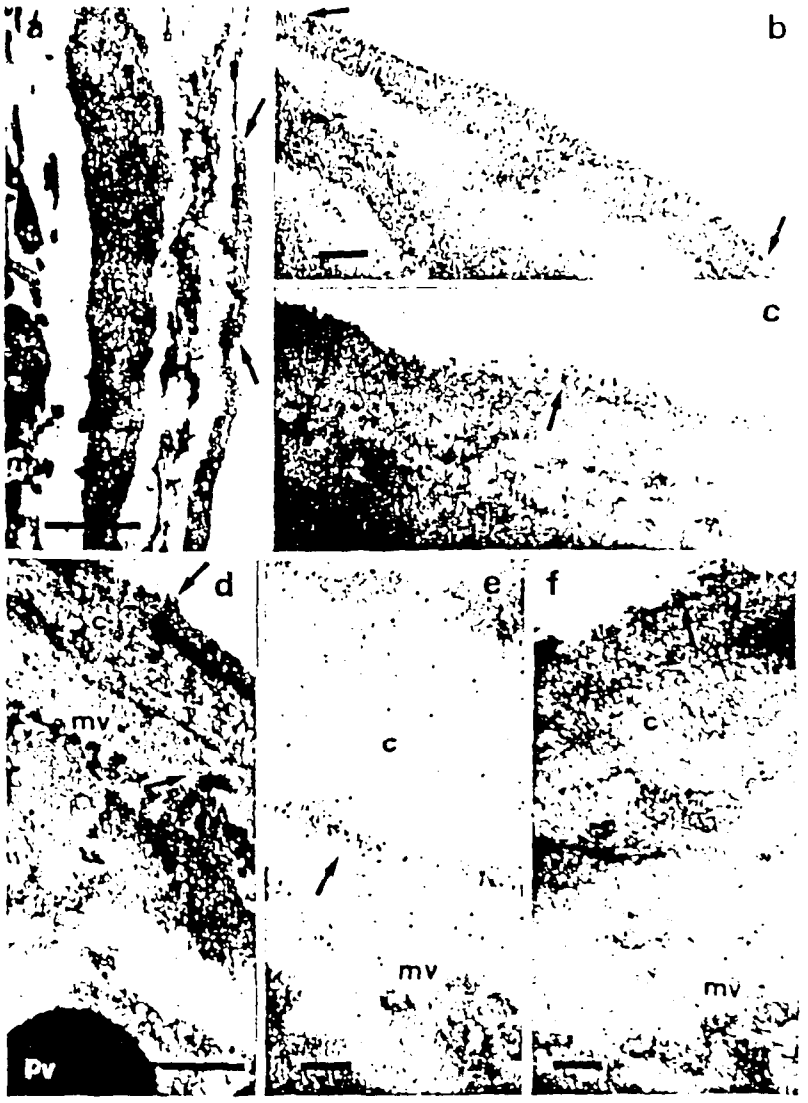


Figure 14. a,b,c. Oocytes injected with BuChEmRNA and muscle poly(A) RNA. a: A very well defined cluster of gold particles (between arrows) located in the external part of the follicular cell (fc) layer. A few gold particles can be seen between the microvilli (mv) and the follicle cells. Scale bar = 5 um. b: Close-up view of (a) - note the specific external localization close to the extracellular matrix. Scale bar = 1 um. c: Small aggregates with the same localization. Scale bar = 1 um. d,e,f. Oocytes injected with BuChEmRNA and brain poly(A)$^+$ RNA. d: Animal pole with pigment vesicle (pv). The gold particles are located in two different compartments: One (arrow) between microvillae (mv) and collagen level (c) and the other in a small cluster on the external part of the follicular cell. Scale bar = 5 um. e,f: Detail of the two accumulation compartments. In (e), most internal compartment and (f), external accumulation. Scale bar = 1 um.

Figure 15. a,b,c,d,e. Oocytes injected with BuChEmRNA after preincubation with intracellulary introduced tunicamycin. a: Animal pole, with pigment vesicles (Pv) and yolk vesicle (Yv); gold particles are highly concentrated in the cytoplasm (arrow) around the yolk vesicles (Yv). The extracellular part of the oocyte is defined by the microvilli (mv). Few gold particles are associated with the extracellular material of the oocyte cell (arrow). Scale bar = 5 um. b: Close-up view of the cytoplasmic area in the animal pole side. Few gold particles are closely associated (arrow) with yolk vesciles (Yv). Scale bar = 1 um. c,d,e: Aggregates of gold particles (arrow) are located around membrane ghost (star). Scale bar = 1 um. Control experiments. f: Oocyte co-injected with BuChEmRNA and muscle poly(A)$^+$ RNA, incubated with PBS and Protein A-gold. This control tests the nonspecific binding of the Protein A-gold on an mRNA injected oocyte. Rare gold particles (small arrows); Basal lamina (BL) is visible on the external part of the follicular cell (fc). Scale bar = 5 um. g: Barth medium-injected oocyte, incubated with PBS and Protein A-gold. This control tests the non-specific binding of the Protein A-gold on an oocyte injected without mRNA. Only a few gold particles are detectable in the extracellular matrix material; collagen (c); the animal pole side. Scale bar = 5 um. h: Barth medium-injected oocyte, incubated with anti-AChE polyclonal antibody and Protein A-gold. The gold particle accumulation in the extracellular matrix associated with the collagen (c), and around the follicle cell (fc), is not negligibl . Scale bar = 5 um.

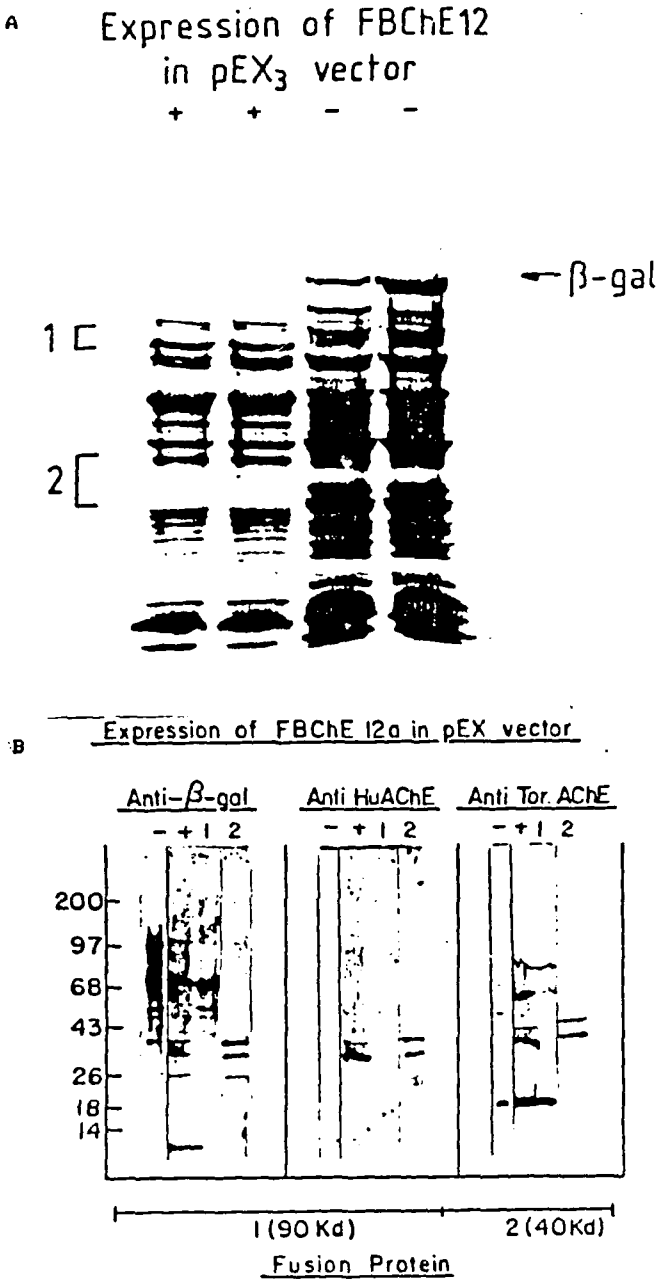


Figure 16. A. Detection of partially proteolyzed fusion protein with beta galactosidase. Ten ul samples of boiled bacterial extracts were subjected to protein gel electrophoresis for 3 hrs and 100 V in gradient slab gels (5-15% polyacrylamide). Bacteria were transformed with pEX$_3$FBChE12 recombinants (+) or with native pEX3 plasmids (-). Protein bands that were found to be enhanced in bacteria infected with pEX$_3$FBChE12 recombinants (Fraction 1, approximately 70-75 kD, and fraction 2, approx. 30-40 kD), were purified and concentrated by preparative gel electrophoresis, electroelution and lyophilization. B. Immunoblot analysis of fusion polypeptide products. The total proteins extracted from recombinant (+) or native (-) pEX3 plasmids and the electroeluted proteins purified from fraction 1 and 2 were electrophoresed and blotted on nitrocellulose filters by overnight electroelution. Immunoblot analysis was performed with rabbit antisera against bacterial beta-galactosidase, human erythrocyte AChE and Torpedo electric organ AChE.

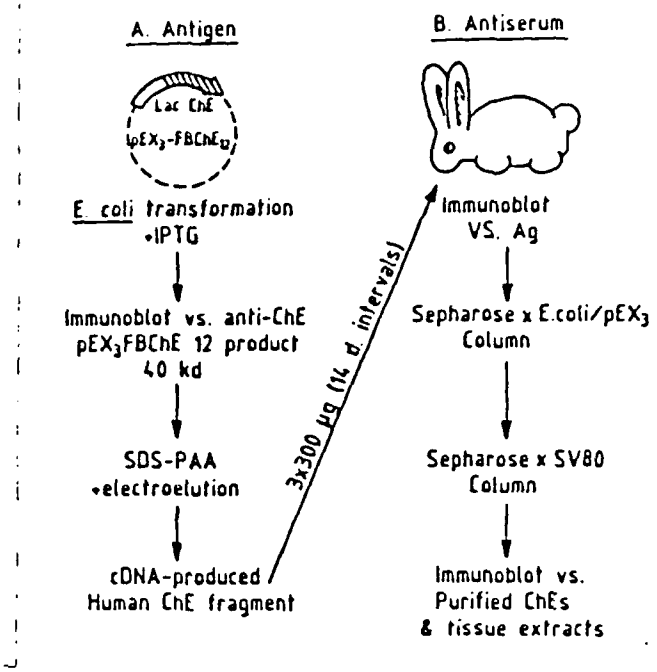
the synthesis of the cro-beta-galactosidase (110 kd) ceased in these bacteria, as compared with its synthesis in bacteria transfected with the original pEX plasmid (108).

The nature of the polypeptides translated from pEX_3FBChE12 plasmids was examined by immunoblot analysis of bacterial extracts (Figure 16b). It was found that all of the proteolytic products derived from the fusion protein reacted with antibodies against bacterial beta-galactosidase. Of these, two peptides of 40 kd and 35 kd in length, but not the heavier ones, interacted specifically in protein blots with rabbit antibodies against AChE from both human erythrocytes and Torpedo electric organ. In addition, the 40-kd and 35-kd polypeptides reacted specifically in immunoblots with all of the AE1-5 mouse monoclonal antibodies raised against human erythrocyte AChE by Fambrough et al. (58). Altogether, the interaction with antibodies indicated that these polypeptides are derived from a partially proteolyzed fusion protein of beta-galactosidase-FBChE12, with the 40-kd and 35-kd polypeptides containing the information encoded by the FBChE12 cDNA insert as well as some parts from the C-terminal domain of beta-galactosidase. Furthermore, the immunoblot analysis revealed that these BuChE-derived polypeptides share immunological properties with both human and Torpedo AChE. Protein blot analysis with antiserum against whole human serum proteins, which efficiently interacts with BuChE in crossed immunoelectrophoresis plates, failed (116) to reveal a significant specific interaction, perhaps because the antibodies interacting with BuChE in this complex antiserum recognize only the mature protein.

b. Anti-cholinesterase antibodies elicited against the protein product of BuChEcDNA as synthesized in bacteria interact with cholinesterases in immunoblots

The 40-kd and 35-kd protein products synthesized in bacteria from the FBChE12 insert were employed to elicit rabbit anti-ChE antibodies (Figure 17a,b). The resultant rabbit serum, purified by affinity chromatography, was tested by immunoblot analysis against the electrophoretically purified antigen. It was also reacted with blotted purified human erythrocyte AChE, as well as with protein extracts from E. coli bacteria transformed with non-recombinant pEX_3 plasmids and from bacteria transformed with pEX_3FBChE12 plasmids. The antibodies interacted specifically with the 70-kd purified AChE, with similarly migrating proteins in serum and muscle, with several additional muscle proteins of variable sizes and with a set of polypeptides from the pEX_3FBChE12-transformed bacteria, the largest of which was ca. 95 kd (Figure 17c). This analysis demonstrated that the anti-cloned BuChE antiserum interacts specifically with the bacterially produced human BuChE and with highly purified erythrocyte AChE, both under complete denaturation and immobilization conditions.

IMMUNOBLOT ANALYSIS WITH ANTIBODIES ELICITED AGAINST THE CLONED FRAGMENT OF HUMAN BUTYRYLCHOLINESTERASE



C Immunoblot Analysis with anti cloned ChE antibodies

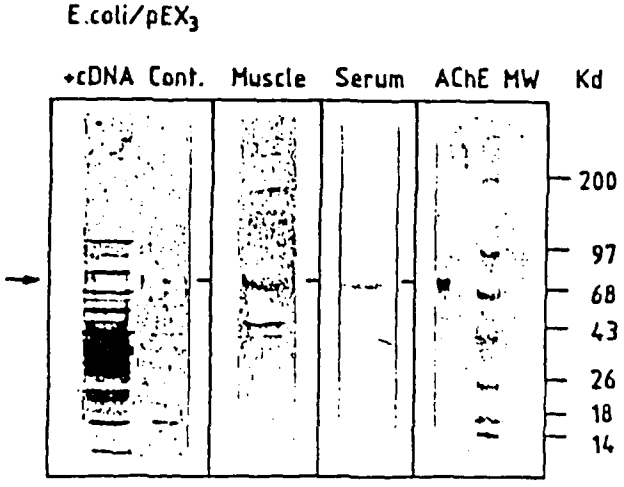


Figure 17. A and B: This scheme summarizes the different steps involved in the elicitation of antibodies from the gene to the immunoblot. Details are given under the section Materials and Methods. C: Antiserum against the fragment of human BuChE expressed in pEX_3FBChE12 plasmids was used for immunoblot analysis in 1:500 dilution. Proteins were extracted from the pellet of bacteria carrying pEX_3FBChE12 recombinants (+) or native pEX3 plasmids (cont.). Highly purified erythrocyte AChE (20 ng, gratefully received from Drs. E. Schmell and T. August, Baltimore) and total protein extract from fetal human muscle were loaded in parallel.

c. Antibodies to clone-produced butyrylcholinesterase interact preferentially with particular globular forms of cholinesterases

The rabbit antibodies directed against the clone-produced BuChE peptides were used for the immunoprecipitation of native and denatured human serum BuChE- and AChE-enriched fractions prepared from red blood cell membranes. For this purpose, serial dilutions of the rabbit antiserum were incubated with the enzyme samples, using immunobeads covered by a goat anti-rabbit IgG (Amersham) as a second antibody. Immunoprecipitated pellets of native ChEs were analyzed for ChE activity (Figure 18). Ca. 15% of the BuChE and 10% of the AChE activities could be precipitated, with significantly higher level of BuChE precipitation and with the same and optimal dilution of the antiserum (1:80) for both activities (108). The denatured ChEs were incubated with ^{3}H-(DFP) and dialyzed prior to the immunoprecipitation reaction, so that the radioactivity recovered in the immunoprecipitated pellets reflected the efficiency of the immunoreaction with denatured ChEs. In this case as well, precipitation curves were obtained for both AChE and BuChE with the same 15-20% level and optimal dilution of the antiserum.

In order to determine which of the various molecular forms of AChE and BuChE from different tissue sources interact with these antibodies, the relevant tissue extracts were subjected to direct immunoreaction followed by sucrose gradient centrifugation and ChE activity measurements (Figure 19). In this experiment, the antibody-reacted serum BuChE tetramers displayed a sedimentation coefficient of ca. 13.5S, as compared with 11.8S for the native enzyme; in contrast, the erythrocyte dimeric enzyme sedimented with exactly the same rate before and after the immunoreaction. Different results were obtained when the complex activities from fetal muscle extracts were similarly immunoreacted. BuChE dimers, but not BuChE tetramers, changed their sedimentation coefficients when reacted with the antiserum. When AChE activities from the same extracts were measured, it was found that here as well, the dimeric form displayed an apparent shift in sedimentation. Small but significant shifts were also detected in the sedimentation patterns of the 10S and the 12S forms of muscle AChE. Thus, the sedimentation rates of AChE dimers from fetal muscle, but not from erythrocytes, and BuChE tetramers from serum, but not from fetal muscle, were significantly affected by these antibodies (Table III).

II.5. Cross-homologies between butyrylcholinesterase and thyroglobulin induce antibodies to neuromuscular junction cholinesterases

Human BuChEcDNA displays a considerable sequence homology with Tg (5,43). The highest level of homology is within the region produced in our pEX bacterial expression system. To examine whether autoimmune antibodies to ChE are induced in hyperthyroid patients, we tested the interaction of Igs from such patients with the protein products of pEX$_3$BuChEcDNA constructs as well as with the enzyme in situ, in fixed muscle fibers. For this purpose, a dot blot immunoreaction with the pEX$_3$BuChEcDNA-derived polypeptides was performed, using diluted patient sera and iodinated protein A.Igs

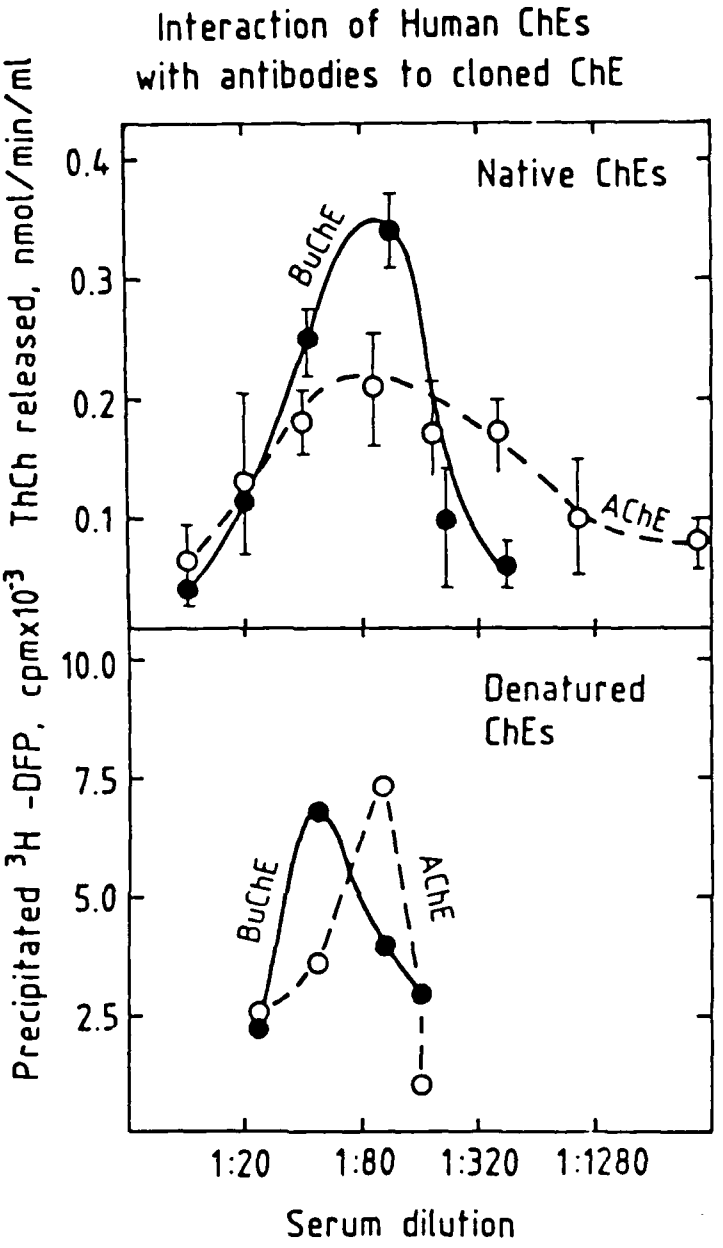


Figure 18. Interaction of human ChEs with antibodies to cloned BuChE. A. Precipitation curves. The rabbit antiserum elicited against cloned BuChE was serially diluted and incubated with enriched preparations of serum BuChE or erythrocyte AChE, either in their native forms (top, avg. of three different experiments and deviations) or following interaction with [^{3}H]DFP and denaturation (bottom). Complexes with the second antibody were formed by further incubation with goat anti-rabbit IgG conjugated to Sepharose beads and precipitated by centrifugation. The extent of interaction was then determined, either by catalytic activity measurements or by counting precipitated [^{3}H]DFP. Control reactions were performed by incubation with non-immune rabbit serum at similar dilutions.

SUCROSE GRADIENT ANALYSIS OF ANTIBODY/ChE COMPLEXES

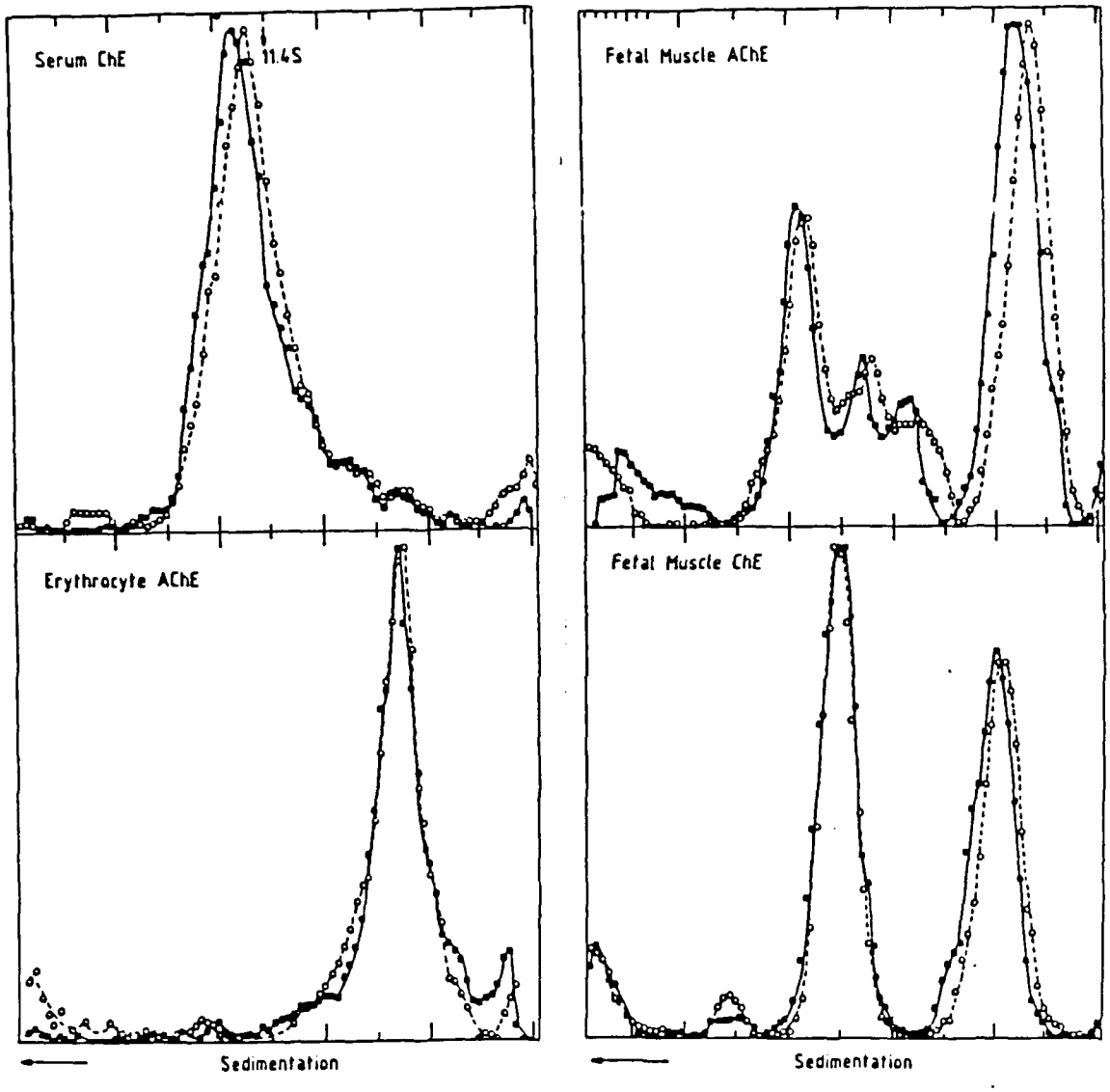


Figure 19. Sucrose gradient sedimentation. Anti-cloned BuChE antibodies were incubated with enriched serum BuChE or erythrocyte AChE, or fetal muscle extracts and ChEs were separated from each of these mixtures by centrifugation on linear 5-20% sucrose gradients (o). For comparison, native ChE forms were run in parallel (o). Bovine catalase (11.3S) served as the principal sedimentation marker.

Altered sedimentation coefficients in specific molecular forms of ChE as induced by antibodies.

	4 to 6S	10S	12S	16S
Serum BuChE	--	--	2S (± 0.2S) hydrophilic	--
Erythrocyte AChE	0	--	--	--
Fetal muscle BuChE	1S (±0.2S) hydrophilic	--	0	A
Fetal Muscle AChE	1.7S (± 0.1S) hydrophilic hydrophobic	1.3S (± 0.1S) hydrophilic hydrophobic	1S (± 0.1S)	A

Table III

0 : no shift

-- : absence of molecular form

A : ambiguous

The shift of the molecular form is indicated in S unit ± the standard error of the mean (s.e.m.) for 3 independant experiments.

from three normal individuals demonstrated nil binding above background. The polyclonal rabbit anti-cloned ChE showed the highest level of binding, as expected. Six of nine patients showed evidence of significant binding to the ChE protein.

Further analyses included immunoblots of the pEX_3BuChEcDNA products following gel electrophoresis (Figure 20). Purified Tg served as a positive control in these experiments. Using these polyclonal antibodies, faint but distinct bands could be detected for Tg with anti-cloned ChE and vice versa. Five normal Igs had undetectable binding to either ChE or Tg. Six Hyperthyroid patients demonstrated binding to ChE but this was not always accompanied by binding to Tg. In three patients with hyperthyroidism, there was no binding to ChE despite high levels of binding to Tg.

II.6. Cholinesterase genes are expressed in the haploid genome oocytes

Dot blot hybridization of poly(A)$^+$ RNA from ovarian tissue samples from several individuals, using ^{32}P-labelled BuChEcDNA, indicated that the level of ChEmRNAs in the mature human ovary is <0.001% of total mRNA (118). However, reproducible in situ hybridization signals were observed in ovarian sections from various individuals and were localized in single cells, identified as oocytes, within follicular structures of different developmental stages (86) (Figure 21). In a semi-quantitative analysis performed on 71 primordial, 14 pre-antral and 20 antral follicles with positively labelled oocytes, the average grain density of hybridization signals on a scale of 1-5 was estimated to be 1-2, 4-5 and 2-4, respectively. Thus the level of BuChEmRNA appeared to be reproducibly high in pre-antral follicles as compared with those which were primordial and antral, while atretic follicles remained negative and other cell types did not display significant labelling (86,118) (Figure 22). No signals could be detected in many follicles, probably because of different sectioning levels, where many sections did not cut through the oocytes themselves. No labelling was observed in control experiments using an irrelevant DNA fragment from a non-expressed intron of the human superoxide dismutase gene (119) or following pre-treatment of the ovarian sections with pancreatic ribonuclease. Over 550 grains could be counted in single oocytes exposed for 15 days, indicating high levels of ChEmRNA within the oocyte. For comparison, similar levels were detected under the same hybridization conditions for the pCO_2 mRNA, representing 15% of the total mRNA content in two-cell sea-urchin embryos (120). Considering the low content of oocytes ($\approx 10^5$ in a mature ovary) in the total number of ovarian cells, this high intensity of labelling is compatible with the 0.001% levels of BuChEmRNA detected by dot blot hybridization and suggests that BuChE is expressed in high levels in the human oocyte throughout its development, with a transient increase in the pre-antral phase.

Biochemical properties of the ovarian ChE were examined by sucrose gradient centrifugation followed by measuring [^{3}H]ACh hydrolysis in the presence of selective inhibitors. In several such analyses, ovarian ChE activity was defined as "true" AChE, sensitive to inhibition by 10^{-5}M of the selective anti-AchE inhibitor BW284C51 and resistant to the anti-BuChE inhibitor iso-OMPA (86). However, careful inspection of

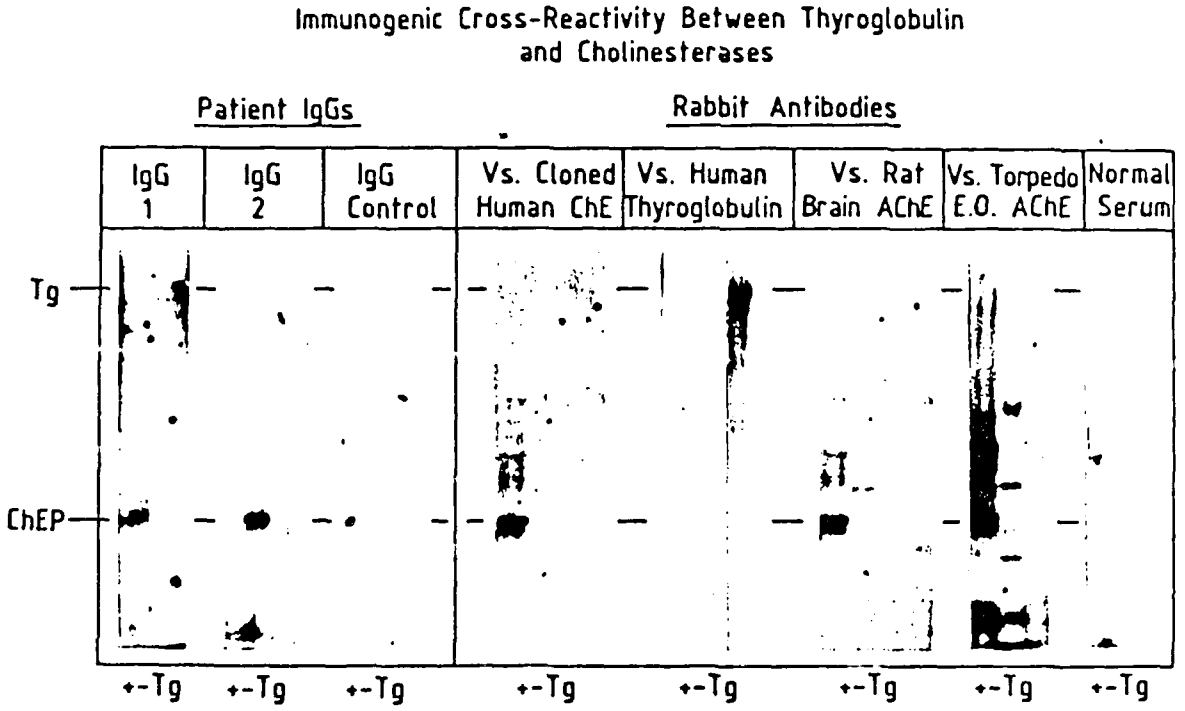


Figure 20. Protein extracts from bacteria transfected with the pEX$_3$BuChEcDNA constructs (+) or with pEX3 plasmids alone (-) were separated by gradient polyacrylamide gel electrophoresis in parallel with purified human thyroglobulin (Tg) and blotted onto nitrocellulose filter. Filters were then incubated with either purified IgG from 2 patients suffering from Grave's ophthalmopathy, with autoimmune antibodies against thryroglobulin, with IgG from a healthy individual (control) or with rabbit antibodies elicited against cloned human ChE, purifed thyroglobulin, rat brain AChE and Torpedo electric organ AChE, as well as with normal rabbit serum. Second incubation was with ^{125}I-protein A. Autoradiography was for 1 week with intensifying screen. Note binding of antibodies to cloned ChE peptides (ChEP) and to thyroglobulin (Tg) bands.

ChE TRANSCRIPTS IN HUMAN OOCYTES

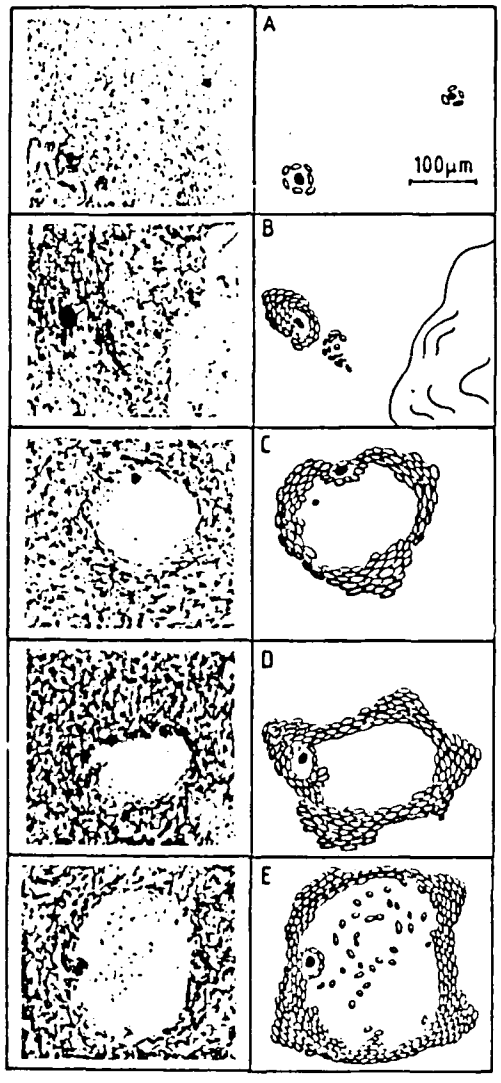


Figure 21. Full-length purified BuChEcDNA was labelled with [^{35}S] deoxyadenosine and deoxycytosine to a specific activity of 3 X 10^8 cpm/microgram and employed as a probe for hybridization <u>in situ</u> with frozen 12 micron sections from mature, normal ovaries. Photographs and drawings of follicles in primordial (A), pre-antral (B), and antral (C-E) stages are displayed. Note absence of labeling in atretic follicle (B, right) and intense labeling in pre-antral follicle (B, left).

ChE LEVELS IN DEVELOPING OOCYTES

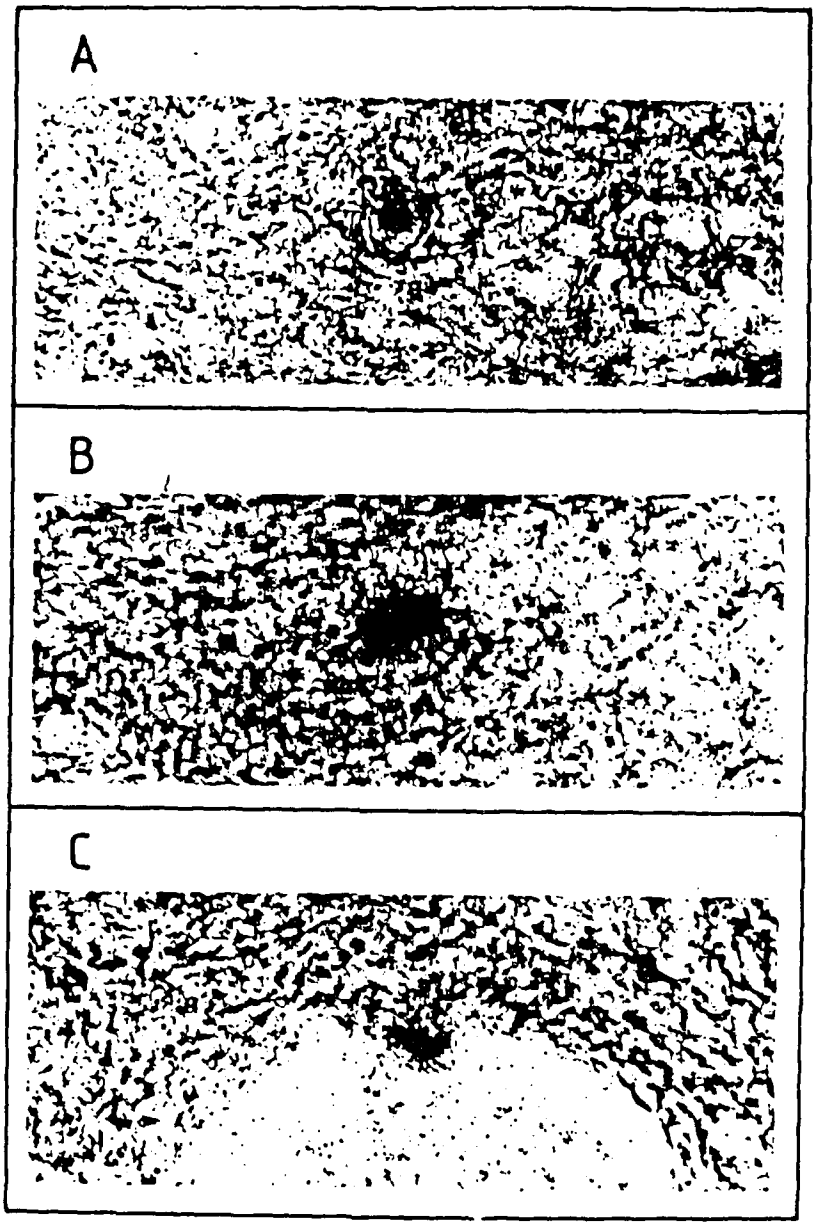


Figure 22. ChEmRNA levels in developing oocytes. In-situ hybridization was as in previous figure. High magnification photographs are displayed for follicles at the primordial (A), pre antral (B) and antral (C) stages. Note relatively intense labeling of the oocytes at the pre-antral stage.

cross-hybridization levels between AChEcDNA and BuChEcDNA revealed high specificity of the hybridization signals (121), strongly suggesting that the in situ hybridization of BuChEcDNA with ovarian sections revealed the exclusive presence of BuChEmRNA. Further experiments using AChEcDNA demonstrated that AChEmRNA is also produced in the oocytes, although in lower quantities (Ayalon, Zakut, and Soreq, unpublished data). It should be noted that the presence of mRNA transcripts does not necessarily imply that their corresponding protein products are present and biologically active. This is particularly true in the case of oocytes, where accumulation of mRNA transcripts for later use after fertilization has been observed. Cytochemical staining experiments will have to be performed on ovarian sections to resolve this issue. The ovarian AChE appeared to be predominantly soluble in low ionic strength buffer, but up to 30% of the activity remained attached to the buffer-insoluble fraction of the ovarian homogenates, from which it could be removed by 0.1% of the non-ionic detergent Triton X-100. The buffer-soluble AChE sedimented as 5.5-6.5S globular dimers, whereas the detergent-extractable enzyme presented the properties of both 4.5S monomers and heavier dimers, indicating that it could reflect contamination with erythrocytes. Minor activities of BuChE tetramers, most probably of plasma origin, could be observed in the buffer-soluble fractions of part of the samples and were completely resistant to BW284C51 but sensitive to iso-OMPA at the above concentrations.

II.7. De novo inheritable amplification of the CHE gene in a family under exposure to parathion

We have studied the possible role of OP exposure in CHE gene amplification in an Israeli family from an agricultural settlement in northern Israel (referred to here as the "H family"). The occurrence of the defective BuChE phenotype in the H family was brought to our attention when one of its members, I.T., suffered from characteristic prolonged apnea (81) following a single intravenous administration of succinylcholine during the course of general anaesthesia. On another occasion a sibling, M.I., fainted while spraying parathion in a cotton field. Examination of serum BuChE activity in all members of this family revealed very low levels of BuSCh-hydrolyzing activity in serum samples from both I.T and M.I., with increased sensitivity to the specific OP BuChE inhibitor iso-OMPA (11) and pronounced resistance to the local anaesthetic Dibucaine (2-butoxy-N-(2-diethylaminoethyl)-4-quinoline carboxamide), all being characteristic of the defective BuChE-type enzyme (1).

To examine whether the expression of the unusual BuChE phenotype was due to alteration(s) at the level of the CHE genes, DNA blot hybridization experiments were performed, using ^{32}P-labelled fragments from the cloned BuChEcDNA as probes. When digested with the enzymes EcoRI and Hind III and probed with full-length BuChEcDNA, peripheral blood DNA from M.I. revealed highly positive restriction fragments of approximately 6.0 kb and smaller (for EcoRI) and 2.5 Kb (for Hind III) (Figure 23). These were absent from DNA from several other members of the H family, specifically including the parents, R.U. and M.O., and the sibling, I.T. (122). This pattern was reproducibly obtained using DNA samples taken at long (6-

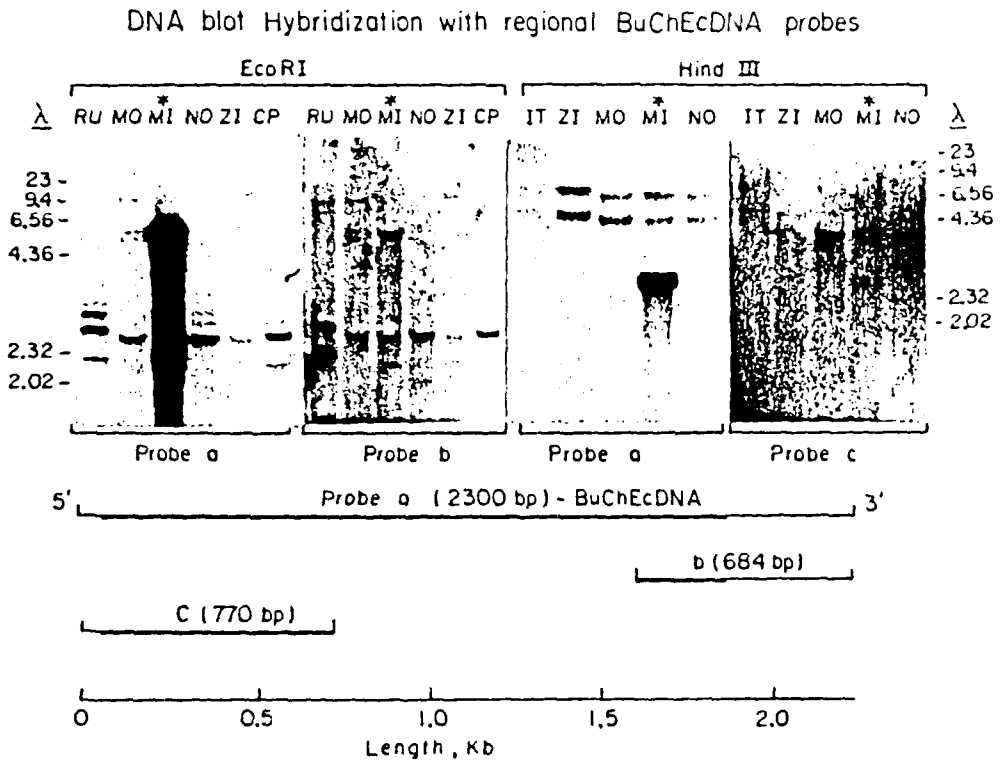


Figure 23. DNA blot hybridization with regional BuChEcDNA probes. 10 ug samples of DNA from peripheral blood were digested to completion with excess amounts of the restriction enzymes EcoRI (Pharmacia) or Hind III (IBI), electrophoresed on 1.0% agarose gel and transferred to a Gene-Screen filter (NEN). Subcloned BuChEcDNA probes a,b and c were prepared as described and labeled with ^{32}P-dATP by the random primer technique. Hybridization was carried out for 16 hr and filters were washed in 0.15M NaCl, 0.015M NaCitrate and 0.1% SDS at 60°C. Exposure was for 6 days at -70°C with an intensifying screen. Lambda phage DNA restricted with Hind III was used for size markers. The probes used are schematically drawn below, with coding regions represented by thick lines and untranslated regions by thinner lines.

month) time intervals. It is interesting that the hybridization signal with these amplified fragments was considerably weaker when the 5' and 3' terminal parts of BuChEcDNA were used as probes, suggesting that the initial amplification event was confined mainly to the central part of the BuChE gene and that the external parts of this gene were amplified to a lesser extent, in agreement with the "onion skin" model for other amplification units as opposed to a pattern of tandem repeats (79). Furthermore, the variable sizes of the positive fragments obtained with EcoRI but not with Hind III also argue against the tandem repeat model for the initial event of the amplification.

To quantitate this amplification, a dot blot hybridization was performed with 6 dilutions of peripheral blood DNA from each member of the H family (Figure 24). DNA from M.I. and one of his sons, O.F., contained an equivalent of about 25 pg of BuChEcDNA-positive sequences per microgram of genomic DNA, whereas DNA from other members of the family displayed levels equivalent to only 1-3 pg/ug DNA. Blot hybridization of DNA from O.F. revealed restriction patterns similar to those of M.I. Altogether, these hybridization experiments suggest that the de novo amplification event occurred in the genome of M.I. very early in spermatogenesis, embryogenesis, or oogenesis, rendering it inheritable. However, we cannot rule out the possibility that O.F. inherited the predisposition for this amplification and that the occurrence of the amplified DNA in his case was due to the prolonged exposure to parathion.

When metaphase peripheral blood chromosomes from M.I. and his mother, R.U., were analyzed by the Giemsa (G) and the bromodeoxyuracil-induced (R) banding techniques (85), apparently normal karyotypes were observed in both individuals, with neither minute chromosomes nor homogeneously stained regions which are commonly found in cases of gene amplifications (80). In situ hybridization with (^{35}S)-BuChEcDNA revealed intense labelling of the 3q29 region in M.I.'s chromosomes as compared with controls. [^{35}S]-Labeling was mostly confined to chromosomal structures, excluding the possibility that the amplified CHE genes were present in submicroscopic extrachromosomal elements.

Altogether, these observations suggested that the inheritable amplified DNA segment was localized close to the original site of the CHE gene at 3q26 (85). Since the amplification unit corresponds primarily to the middle third of the cDNA, and assuming that it is present at a single site in the genome, we calculate that at least 100 copies of the amplified fragment are present in a possibly fully inheritable form in the genomic DNA of M.I.

In spite of the apparent gene amplification in M.I. and O.F., gel electrophoresis and immunoblot analysis of serum proteins with anti-BuChE antibodies failed to reveal over-expression of serum BuChE in these individuals (123). However, this does not exclude the possibility that the amplified gene was over-expressed early in development, for example, in germline cells and embryonic tissues, where the CHE gene is intensely expressed (83,86).

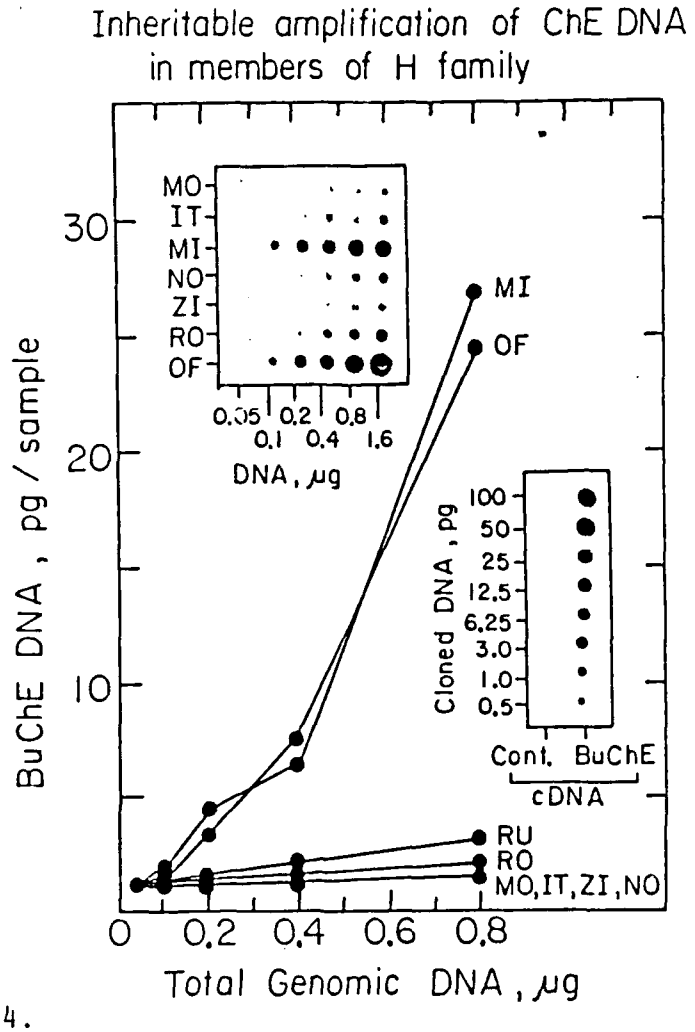


Figure 24.

De..atured genomic DNA from peripheral blood cells was spotted onto a Gene-Screen filter using a dot blot applicator (BioRad). Electroeluted BuChEcDNA (probe a, upper panel) was spotted in parallel for calibration. All samples contained the noted quantities of genomic DNA and denatured herring testes DNA to yield a total amount of 2 ug DNA per spot. Hybridization, wash and exposure were done with (^{32}P)-labeled probe a, as detailed in upper panel. Quantities of genomic BuChEDNA sequences that hybridized with the labeled probe in each member of the H family were determined in values equivalent to pg of BuChEcDNA by optical densitometry of the exposed X-ray film (Agfa Gevaert) in a Bio-Tek microplate reader. An irrelevant clone served as control. Insets: autoradiographed films showing dot hybridizations of ug of genomic DNA (upper) and pg of cloned DNA (lower).

II.8. Co-amplification of human acetylcholinesterase and butyrylcholinesterase genes in blood cells

In order to search for putative structural changes in leukemic DNA samples within the human AChE and CHE genes encoding AChE and BuChE, we first examined their restriction fragment patterns in peripheral blood DNA from 16 patients with various leukemias as compared with DNA from 30 healthy individuals. For this purpose DNA blot hybridization was performed with equal amounts of patients' DNA following complete digestion with the restriction endonucleases PvuII and EcoRI and gel electrophoresis. Hybridization with [32p]-labelled AChE cDNA and BuChE cDNA repeatedly revealed invariant restriction patterns and signal intensities for DNA from all of the healthy individuals. The same restriction pattern and signal intensities were observed in DNA from 12 of the leukemic patients. In contrast, the hybridization patterns in the 4 remaining samples displayed both qualitative alterations and a clear signal enhancement with both cDNA probes (121). It revealed intensified labelling of bands that also existed in the control lane, as well as the appearance of various novel labelled bands (Figure 25a).

In view of these first promising results and the previous reports correlating ChEs with megakaryocytopoiesis and platelet production (87-89), we examined DNA from additional patients with platelet disorders, whether or not defined as leukemic. Significantly enhanced hybridization signals with both cDNA probes were found in 3 of 5 such patients examined, one of them leukemic (Figure 25b). It is interesting that the intensity of hybridization in 2 of these samples was much higher than it was in any of the previously tested leukemic DNA samples. Furthermore, the amplification events in these two samples appeared to involve many additional PvuII-cut DNA fragments, due to either nucleotide changes producing novel PvuII restriction sites or to different regions of DNA having been amplified (121).

To further compare the restriction fragment patterns of the amplified genes, we subjected the relevant lanes from these autoradiograms to optical densitometry (Figure 25c). This analysis clearly demonstrated the appearance of slightly enhanced hybridization signals at equal migration positions to those observed in control DNA for a representative leukemic DNA sample with a moderate amplification. In another leukemic DNA sample taken from a patient with reduced platelet counts, the densitometry signals were higher by an order of magnitude and presented several additional short PvuII-cut fragments. Yet much higher signals and more novel bands of various sizes were observed with a third sample, derived from a non-leukemic patient with a pronounced decrease in platelet counts (thrombocytopenia).

The variable degrees of amplification occurring in the genes coding for AChE and BuChE in these individuals were quantified by slot blot DNA hybridization, using a 5-fold dilution pattern. Cross-hybridization between the two cDNA probes was exceedingly low (less than 0.01), demonstrating that the observed amplification events indeed occurred in each of these genes and did not merely reflect similarity in their sequences. One microgram of genomic DNA from the patients with CHE and ACHE gene amplifications included genomic sequences

AMPLIFIED CHE GENES IN ABNORMAL BLOOD CELLS

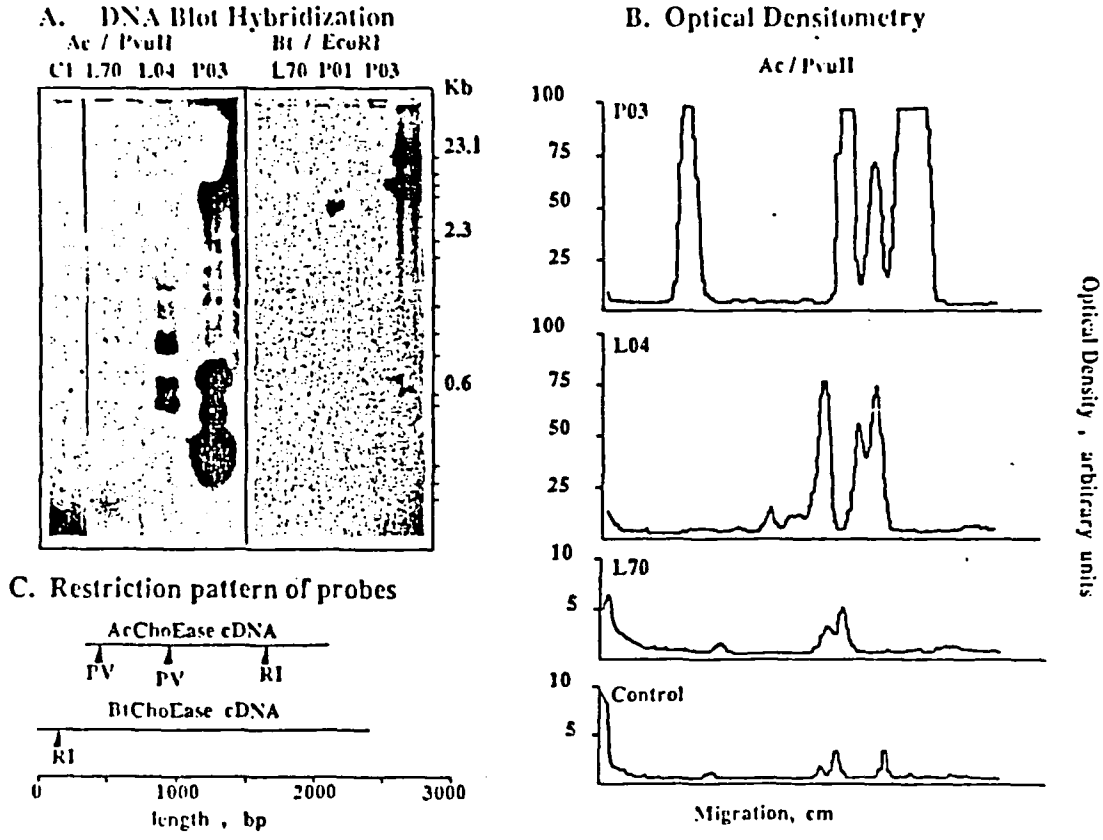


Figure 25. Comparative analysis of representative DNA samples from a healthy control (C1), a leukemic AML patient with moderate amplification (L04), and a nonleukemic patient with a pronounced decrease in platelet counts (P03) was performed by blot hybridization analysis using ^{32}P-labelled probes. (A) Blot hybridization with Pvu II-cut genomic DNA and AChEcDNA probe (Ac) and with EcoRI-cut genomic DNA and BuChEcDNA (Bt). Optical densitometry of individual lanes from the Pvu II-treated AChEcDNA-hybridized blot was performed at 545 nm. (C) Restriction sites for Pvu II and EcoRI on the cDNA probes. Exposure was for six days.

equivalent to about 0.1 and 0.01 ng of the purified BuChEcDNA and AChEcDNA inserts, respectively. Parallel analysis using similar quantities of control DNA revealed considerably lower signals with both probes.

Taking the total complexity of human genomic DNA as $4 \cdot 10^9$ base pairs, this implied that at least 40-100 copies of both these sequences are present in the above DNA. Other examined DNAs featured about 10-fold lower signals with BuChEcDNA, reflecting a more modest amplification on an order of up to 20 copies per genome (121). Repeated hybridization of the same blots with a cDNA probe coding for a rat ribosomal protein, a non-related gene for a structural protein, demonstrated no amplification in all of the examined samples and similar labelling intensities for both patient and control DNAs.

Altogether, 6 cases of co-amplification within the ACHE and CHE genes were observed in DNA samples from 20 patients with abnormal hematocytopoiesis, while DNA from 30 healthy individuals showed neither amplification nor polymorphism with respect to the restriction patterns obtained with these probes. The DNA samples presenting these amplifications were derived from 4 cases of AML with 10-50 copies of both ACHE and CHE genes, and 3 cases of platelet count abnormalities. One expressed excess platelets count and 10-20 copies of the ACHE and CHE genes, and the two others showed reduction of platelets count and featured 10-200 copies of the same genes. These striking concomitant multiplications presented a highly significant correlation ($p<0.001$) between amplification of CHE-encoding genes and the occurrence of abnormal myeloid progenitor cells or promegakaryocytes in the examined individuals.

III. Discussion and Conclusions

III.1. Biochemical implications to sequence similarities within the cholinesterase family

Cholinesterases have a catalytic mechanism similar to that known for the serine proteases (reviewed in ref. 100). In line with the widely accepted notions that functional similarity reflects common ancestral genes and that multigene families have developed by gene duplication and subsequent divergence during evolution (124), Augustinsson (125) suggested that ChEs are phylogenetically related to the large family of serine proteases and may be defined as members in the multigene family of serine hydrolases. The 3 out of 8 match in the concensus sequences of the catalytic sites of carboxylesterases and serine proteases, including the invariant serine residue, further suggested a common origin of these two families (126). Recent molecular cloning and DNA sequencing studies confirmed the phylogenetic relationships within the gene families of serine proteases (127) and of carboxylesterases (128), but left the question of their inter-relationships open.

Profile analysis of the human AChE amino acid sequence showed no similarity to any of the ≥4500 protein sequences in the European Molecular Biology Laboratory (EMBL) protein data base, with the exceptions of Torpedo and Drosophila AChEs, human BuChE and bovine Tg. Human AChE did not specifically show any resemblance of serine protease sequences. In view of the sequence information deduced from cloned DNAs for four different ChEs, and based on the above-discussed arguments, it now appears that human AChE joins the other ChE species to form a limited minigene family that belongs to the larger family of carboxylesterases type B but appears to be distinct from the other serine proteases. Our analysis therefore extends and supports the recent conclusion of Richmond and colleagues (96) in suggesting that ChEs cannot be included in a serine hydrolase multigene family.

Within the ChE family, the high sequence similarities between human AChE and BuChE imply that variations and conservations in the primary amino acid sequence of ChEs may be implicated with distinct differences in the substrate specificity and sensitivity to selective inhibitors that were observed for particular types of ChEs. Detailed analysis of these sequences by site-directed mutagenesis and expression of the modified genes in heterologous systems may therefore reveal the key residues in the charge-relay system of ChEs and lead to the development of improved therapeutic drugs against OP intoxication (129).

The complete amino acid sequence of BuChE reveals some characteristics which are of interest for understanding the biological properties of this protein. The first 70 amino acids represent a fully open reading frame containing a leader peptide which may be cleaved off in the course of biosynthesis and allows a transmembranous transfer of the nascent protein during its translation and post-translational processing. However the possibility should be considered that this peptide remains in the complete, mature BuChE molecule, where it serves

as a membrane anchor similar to that of the asialoglycoprotein receptor (106). Further consensus sequences within the BuChEmRNA transcript reflect the occurrence of glycosylations, taking place within the Golgi apparatus. The sequence also shows seven cysteine residues, among them six that are probably implicated in intramolecular S-S bonds. The remaining cysteine could be involved in the inter-subunit S-S bond that covalently binds BuChE catalytic subunits to each other (68). It is interesting that the AChE sequence is considerably richer in cysteine residues, which might indicate a different intramolecular folding of its polypeptide backbone. The serine residue in the active esteratic site, a major characteristic of the enzyme, has been conserved in all of the members of the ChE gene family.

Screening of several cDNA libraries from various tissue origins resulted in the isolation of identical cDNA clones, all coding for serum BuChE. These were apparently transcribed from the same gene and were processed similarly in both fetal and adult tissues. These findings indicate that the human CHE gene, encoding serum BuChE, is most probably transcribed into a single transcription product in all tissues. In view of the low number of BuChEcDNA clones in the adult liver library, we cannot as yet exclude the possibility that alternative splicing is involved in the regulation of this gene in humans, similarly to its use in AChE production in Torpedo (103,130). However, our present findings imply that alternative splicing is not responsible for the heterogeneity observed in liver and brain BuChE forms. In the absence of indications for the involvement of transcriptional control, post-transcriptional mechanisms can be pursued as an origin for BuChE heterogeneity. Therfore, oocyte microinjection experiments were initiated with clone-produced synthetic BuChEmRNA.

III.2. Post-transcriptional regulation of cholinesterase heterogeneity

When injected into Xenopus oocytes, synthetic mRNA transcribed from a cDNA encoding human serum BuChE induced the production of a protein displaying the substrate specificity and sensitivity to selective inhibitors characteristic of native BuChE, and which clearly distinguish it from AChE. These results indicate that the ligand binding specificities of BuChE reflect a property which is inherent to the primary amino acid sequence of the molecule. The level of mRNA expression achieved in the oocytes using pure synthetic message is relatively low, when compared with the 10^5-fold enrichment of BuChEmRNA over that observed in tissue extracts (43,46). This suggests that the injected BuChEmRNA is encountered with competing endogenous oocyte mRNA's for the limited number of rough endoplasmic polysomes in the oocyte (47). Alternatively, or in addition, it may reflect limited distribution of the injected RNA within the oocyte.

The 10-fold increased IC_{50} toward iso-OMPA observed for the membrane-associated enzyme suggests that interaction with detergent at the extraction step alters its hydrophobic character, which reduces its affinity for this OP ligand. Similar alterations occurred when detergent was added to the soluble oocyte fraction (131r). However, the Km for BuSCh does not appear to be altered by detergent association. The observation that the single mRNA template employed directed the

synthesis, in oocytes, of catalytically active BuChE displaying native ligand binding affinities could exclude post-translational modifications as a primary determinant of the 3-dimensional structure of the active site and suggests that the variable BuChE phenotypes, like the "atypic" or the fluoride-insensitive serum enzymes, reflect mutations altering the amino acid sequence of these enzyme forms. Indeed, blockage of glycosylation by tunicamycin although impairing transport of the enzyme to the membrane, did not seem to interfere with its catalytic activity. It should be noted that such changes should not necessarily occur in the active site region, as indeed is the case for the "atypic" enzyme (10).

Taking the average membrane-associated activity as 8.1 umole BuSCh hydrolyzed/hr/oocyte implies 1.2×10^{15} molecules BuSCh hydrolyzed/sec/oocyte. Assuming a turnover rate of 1×10^{4} molecules/active site/sec (132) implies 1.2×10^{11} catalytic sites/oocyte in the membrane-associated fraction, or 6×10^{10} dimeric BuChE molecules/oocyte. In an oocyte injected with synthetic BuChEmRNA alone, approximately 2000 sq. microns, or 0.01% of the oocyte surface was occupied by high-density immunoreactive ChE molecules (133). Outside these areas the density of BuChE molecules on the cell surface is therefore suggested to be below the 1×10^{3} molecules/um^2 threshold required for detection using immunohistochemical techniques (134). We therefore assume that the non-fluorescent surface area contains undetected BuChE molecules evenly distributed at a concentration 10-fold lower than that found in the immunoreactive loci. Assuming that the total external surface area of the oocyte actually includes the theca and follicle cell layers as well as the enlargement contributed by the micro- and macrovilli of the oocyte and follicle membranes (135,136) yields a 100-fold enlargement of the total surface area sequestering BuChE and allows us to consider a factor of 10^{5} in the relative area occupied by undetected as opposed to immunostained ChE. Therefore, the molecular density of ChE in a cluster or patch can be estimated to be:

$6 \times 10^{10} = 2 \times 10^{3}(X) + (0.1X)(2000)(10^{5})$ or,

$(X) = 3 \times 10^{3}$ molecules/sq. micron

It is interesting to note that both the nicotinic ACh receptor (134,137) and AChE in neuromuscular junctions and along neuronal dendrites (138, 23, 139) were estimated to be aggregated at molecular densities within the same order of magnitude. This observation might indicate that the organization of membrane-bound molecules within the extracellular surface reflects a precisely regulated physiological property of the involved subcellular structures which is conserved through evolution.

Microinjected alone, synthetic BuChEmRNA induced the formation of primarily dimeric ChE. This first level of oligomeric assembly may therefore be spontaneous, or may require a catalytic mechanism that is already available in the oocyte. It should be noted in this respect that the detection of small, but detectable, AChE activities in Xenopus oocytes (47) and high levels of BuChEmRNA in human oocytes (86) indicates that ChE represents a natural endogenous oocyte protein. The observation that tissue-extracted mRNA's induced higher levels of multi-subunit assembly - possibly including the

incorporation of non-catalytic subunits - indicates that additional protein species, not available in the oocyte, are required to direct the biosynthesis of more complex molecular forms. Given the high degree of tissue-specific polymorphism of ChE molecular forms, it is not surprising to find these additional factors expressed in a tissue-specific manner. Nonetheless, the nature and number of these factors remains to be elucidated.

Supplementation with tissue mRNAs increased the number and intensity of patches and clusters, indicating the induction by tissue-specific factors of enhanced BuChE aggregation at the external surface of the oocyte. This property of enhanced aggregation seems to be correlated to the appearance of molecular forms containing 4 or more catalytic subunits, and may reflect the provision of tissue-specific, membrane-anchoring elements. Such elements were found to be required for the aggregation of both the nicotinic ACh receptor and AChE at the neuromuscular junction (34,140,141), and it would be intriguing to reveal whether the situation here is parallel.

Although both brain and muscle mRNA's induced both patches and clusters, the relative distribution of each type of formation varied between oocytes co-injected with mRNA from the two tissue types in a manner consistent with the organization of ChE in the native tissues (140-142). Furthermore, the relative staining intensity of clusters and patches obtained with muscle mRNA qualitatively exceeded that obtained with brain mRNA. Together, these observations imply a qualitative and/or quantitative difference between ChE-related mRNA's in different tissues and that these mRNA pools are capable of modulating tissue-specific usage of a single BuChEmRNA species.

Several classes of membrane-anchoring elements have been considered in conjunction with AChE. Considerable evidence implicates covalently linked glycolipid, most prominently phosphatidylinositol, as a principal mechanism for the anchorage of globular dimeric AChEs in plasma membranes (4,143). In addition, non-catalytic subunits and proteoglycans have been implicated in the attachment of ChE to membranes in the brain (33) and to the basal lamina at the neuromuscular junction (15,144,145). We do not yet know which of these mechanisms, if any, is operative for BuChE in our system. However, the recent suggestion that alternative mRNA processing is involved in the membrane attachment of hydrophobic AChE in Torpedo (103,146,130) does not appear applicable here, since a single mature BuChEmRNA induced a complete array of molecular forms in the oocytes including soluble and non-soluble pools.

Taken together, these findings demonstrate that both muscle and brain express mRNAs encoding peptides which: 1) are required for the biosynthesis of BuChE molecular forms consisting of multiple dimeric units, 2) may specify the incorporation of non-catalytic subunits, 3) direct the formation of a tissue-specific array of BuChE molecular forms and 4) direct a tissue-specific organizational pattern of BuChE at the external surface of the injected oocytes.

The conspicuous intracellular accumulation of enzyme induced by tunicamycin indicates that post-translational glycosylation plays

an essential role in the transport of BuChE to the external surface. The unequal animal-vegetal pole distribution of induced BuChE in the oocytes indicates an active, polar assymmetry which has been described previously for morphological characteristics such as the yolk platelets or cytoskeletal elements (135,136,147,148) and also for a maternal mRNA localized in the vegetal hemisphere (149). Our present findings suggest that the polar buildup of the oocyte cytoskeletal elements takes an active part in directing newly synthesized proteins to their ultimate site of association at predefined extracellular positions in a manner similar to other polarized cell types (150) and that the animal pole of the Xenopus oocyte is preferentially designated for deposition of nascent surface-associated proteins.

III.3. Immunochemical implications of the similarity and heterogeneity between cholinesterase forms in various tissues

BuChE represents one member of a family of enzymes. During the course of this work, we have also examined its relationship to the other member of this family, AChE, by immunological criteria.

For this purpose, we have used peptides included in the N-terminal 200 amino acids of human BuChE as an antigen. This part of the enzyme may be distinguished both by its particularly low immunogenicity, as revealed by the computer prediction according to Chou and Fasman, (110,112), and by its considerable sequence homology to other ChEs (5). The cDNA-produced BuChE peptides, having been produced in bacteria, are not glycosylated. Furthermore, these polypeptides include only part of the cysteine residues forming the S-S bonds in the complete BuChE molecule, and their tertiary structure is, most probably, different from that of the native protein.

We proceeded to elicit antibodies against these naked, non-processed peptides, with the following assumptions in mind:

1. The anti-cloned ChE antibodies might have novel properties, not detected previously, since conventional antisera, induced against the mature, fully folded enzyme, would not include antibodies against the low immunogenicity N-terminal part of BuChE as major species.
2. Antibodies elicited against this particular part of the enzyme would tend to have a low titer to the antigen because of its low immunogenicity, and might display low affinity because of the complete lack of processing of these peptides in bacteria.
3. Such antibodies would interact with particular forms of the native, non-denatured enzyme only if the epitopes within the N-terminal polypeptide would be at the external surface of the antigen molecules and accessible for immunoreaction.

Consequently, these domain-specific antibodies could be useful for the detection of both sequence homologies and structural differences between various molecular forms of ChEs, in spite of their expected low affinity binding and low titer. The heterologous patterns of immunoreactivity obtained in this study confirm the validity of this assumption.

When tested by immunoblot analysis, the BuChE peptides produced in bacteria interacted efficiently with antibodies reported to be selective for AChE (55,59); vice versa -- the anti-cloned ChE antibodies interacted efficiently with the blotted fully denatured forms of both BuChE and AChE from blood. In contrast, their ability to precipitate the native or even denatured enzyme from solution was rather poor and with higher specificity towards BuChE, in agreement with the above-noted expectations. Even in the presence of a second antibody, which binds all of the Ig molecules present in the immunoreaction mixture, an equivalence zone of 1:80 dilution was determined. Assuming that the concentration of the antibody in the equivalence zone is close to the Kd, this dilution implies that the concentration of active antibodies in raw antiserum is about $1x10^{-7}$ M. These results could reflect the low immunogenicity of the N-terminal part of the BuChE molecule, as predicted by computer analysis.

About 10 to 15% of the total activity could be precipitated under these dilution conditions. Because the rabbit immunization was performed with a denatured ChE, produced in bacteria from a cDNA clone, the specificity of the immunoreaction was also examined in solution with the denatured purified enzyme, previously labeled by ^{3}H-DFP. The antibody titer remained equal to that observed with the native enzyme, confirming that there is a very low concentration of the specific antibody.

Sucrose gradient centrifugation revealed that the antibodies interreacted specifically with BuChE tetramers from serum and BuChE dimers from fetal muscle. They also bound to dimers and tetramers of AChE from fetal muscle. However, there was no significant binding to the AChE dimers from the erythrocyte membrane or to tetramers of BuChE from fetal muscle. The common element to sedimentation peaks which include ChE molecular forms that interact with the antibodies is that they all contain hydrophylic forms. Furthermore, the extent of precipitation in muscle extracts could correspond to the fraction of hydrophylic forms within these particular peaks. These results therefore suggest that our polyclonal antibodies are able to recognize epitopes that are common to or exposed for immunoreaction, only in hydrophylic ChE molecular forms.

The antibody-induced alteration of the sedimentation coefficient observed in our gradient analyses (2S) was rather low compared to the values given in the literature (about 3S; 55,58,59,151,56). This finding as well is in accordance with the low titer of the antibody for the serum BuChE, as only 15% of the molecules were shifted. It is impossible to detect two peaks of reacted and non-reacted enzyme molecules on a gradient where the standard deviation is of the same order of magnitude as the shift. In fetal muscle extracts, the dimer peak alone represents 45% of the total activity; thus the proportion of antibody-bound enzyme is higher in this form, which explains its clearer shift.

In addition to their high efficiency in immunoblots, the anti-cloned ChE antibodies were also used for labeling the bound enzyme in muscle fibers. Several lines of evidence suggest that the regions

labelled by these antibodies in crushed muscle fibers are indeed the end plate ones:

1. In all of the fibers analyzed, we never found more than one labelled region per fiber, which is in accordance with the monofocal innervation of human skeletal muscle.
2. The morphology of the binding region, a very simple "plaque," suggests single gutter structure without secondary foldings, which corresponds to the premature neuromuscular junctions that one expects to observe at this developmental stage.
3. In all cases, labeling coincided with intensive cytochemical staining for ChE activity, as expected for end plate regions. Based on this evidence, the in situ labeling of slightly fixed crushed fetal muscle fibers using these antibodies suggests that the N-terminal part of human BuChE or a peptide highly homologous to it is present in an immunoreactively exposed form in the fetal junction.

Although we cannot conclude whether the in situ binding occurs with AChE or BuChE, both of which are highly concentrated in end plate regions, it is interesting to note the correspondence between immunoreactivity and ChE activity at the neuromuscular junction.

In conclusion, the experiments performed with antibodies elicited toward the N-terminal part of BuChE as expressed from cloned cDNA in bacteria suggest the existence of extensive sequence homology in this part of human ChE between various forms of AChE and BuChE in different tissues. Previous reports of the lack of cross-reactivity between antibodies to AChE and BuChE could hence be explained by the low immunogenicity in this part of the molecule or be due to structural differences between various molecular forms of the enzyme within and between tissues. It should be noted in this respect that the amino acid sequence of human AChE is currently being determined in our laboratory (9), and that this conclusion has been fully confirmed by the data tranlated from the cDNA coding for AChE.

Our findings also point out that similarities in substrate specificity and molecular form composition (such as those between BuChE tetramers in blood and BuChE tetramers in fetal muscle) do not exclude the possibility of structural differences between such homologous forms. This is in agreement with our recent in situ hybridization studies, in which we found three different sites on human chromosomes which carry BuChE coding sequences (85). Primary sequence differences would explain the heterologous immunochemical properties of highly similar ChEs and emphasize the importance of multigenic origin and post-translational processing to the final buildup of the polymorphic ChEs in different tissues and body fluids.

III.4. Anti-cholinesterase antibodies in hyperthyroid disease may be implicated in Graves' Ophthalmopathy

The interaction of the polyclonal antibodies raised against the N-terminal part of cloned human ChE with Tg clearly demonstrated that cross-reactivity exists between antibodies to these proteins in their reduced and denatured state. Furthermore, we have

demonstrated that immunoglobulins from patients with GO will bind to ChE in a variety of conditions. Anti-ChE antibodies did not always correlate with anti-Tg activity in the protein blot experiments, contrary to preliminary results reported using an ELISA in which there was a strong correlation between the two (69,65). However, it is important to note in this respect that the antibodies used in the present study were raised against a naked, non-glycosylated part of the human BuChE protein, whereas those tested by ELISA were elicited against the mature, fully glycosylated AChE from Torpedo. It is notable that binding to Tg was not always detected, even amongst patients with higher titers of anti-Tg antibodies as measured by ELISA. It may be that an epitope, recognized by these antibodies, is either prominent in the clone-produced part of the human enzyme or modified in the reducing conditions of the SDS-PAGE.

Not all patients with GO have detectable levels of anti-Tg antibodies and yet two patients in this study who were anti-Tg negative by ELISA bound to ChE in a Western blot. These sera may contain antibodies with a low affinity for Tg but of higher affinity for BuChE. Consequently, they bind to BuChE despite being beyond the limits of detection using Tg.

The immunolocalization studies show that the binding to ChE observed in patients with GO is not merely an in vitro phenomenon. Previous attempts to demonstrate antibodies to eye muscle using a variety of methods, both in vitro (152) and in situ, have been largely unsuccessful with the exception of Mengitsu et al. (153), who have shown diffuse cytoplasmic staining using GO sera by immunofluorescence.

We have focused on formaldehyde-fixed end plate regions of muscle, since the antiserum used was shown, as detailed above, to interact with blotted denatured ChE and because cholinesterase is present at higher concentrations in these areas, although some forms of ChE are also found extra-junctionally. In fetal muscle, the basal lamina is not fully mature, so that ChE is more accessible for antibody binding. Clearly the experiment would be more conclusive using extra-ocular muscle, although this poses some technical difficulty because of the limited quantity of such tissue available.

If indeed anti-ChE antibodies are implicated with ocular muscle pathology, this indicates that ChEs are important for the normal development and/or functioning of various cell types. Another aspect of this conclusion is reflected in the cases of the H family and the leukemic DNAs, where amplification of the CHE gene was observed (see III.6 and III.7).

III.5. Expression of cholinesterase genes in haploid genome suggests an involvement in germ line cells' development and/or functioning

The continuously high expression of AChEmRNA throughout oocyte development suggests that the enzyme may be required for oocyte growth and maturation processes. Inositol 1,4,5-triphosphate, which mimics muscarinic response in Xenopus oocytes (75), triggers the progesterone-

induced activation of amphibian and starfish oocytes. AChE in Xenopus oocytes is seasonally regulated inversely to the reproductive cycle (47), similarly to the muscarinic receptors in these oocytes (154). Damage to the entire cascade of the cholinesterase gene family in nematodes is lethal to the animals (155). Altogether, this may indicate an involvement of cholinergic responses in the process leading to meiotic maturation of oocytes in a selected group of antral follicles. Alternatively, or in addition, the enhanced transcription of ChE genes in oocytes may reflect the accumulation of AChEmRNA for later use during the post-fertilization processes, similar to the accumulation of excess histone mRNAs in developing sea-urchin oocytes (156). This is in good agreement with recent findings demonstrating transiently enhanced BuChE activity early in chick embryogenesis (83,84). Finally, it is possible that AChE is involved in sperm-egg interaction or post-fertilization mechanisms, as suggested from the cholinergic induction of polyspermy in sea-urchin oocytes (71). Transfection experiments or transgenic mice elicitation using ChEcDNA clones (43) may assist in the search for the specific role(s) of AChE in fertilization processes.

The pronounced synthesis of BuChE transcripts in oocytes suggests that the CHE genes in humans are particularly good candidates for the formation and re-insertion of inheritable processed genes. Further characterization of these genes will be required to correlate between specific copies of CHE genes and the particular ChEmRNA transcripts produced in the developing oocytes.

III.6. Hereditary "silent" CHE gene amplification: Putative response to organophosphorous poisoning?

The role of BuChE in embryonic cells is totally unknown, although its involvement in cell and growth division has been suggested. This could possibly indicate that the amplification of the CHE gene might have given a growth advantage to the M.I. embryo, similar to amplified oncogenes in tumors. However, the nucleotide sequence of the BuChEcDNA does not resemble any of the known oncogenes or growth factors. The most likely explanation for the amplification event is therefore related to the ACh-hydrolyzing activity of BuChE.

In an otherwise normal embryo, over-production of normal BuChE might interfere with cholinergic function and be lethal to the developing embryo; in contrast, over-expressed "silent" BuChE would be less harmful because of its very low catalytic activity while perhaps improving the resistance of a developing sperm cell, oocyte or embryo to OP poisoning. We have found that both of M.I.'s parents were working in agriculture when M.I. was conceived, being exposed to high levels of parathion. When combined with the occurrence of a "silent" CHE gene, such exposure may have created conditions under which only the amplification and over-production of "silent" BuChE would permit survival. This event could be related to the extent of exposure, perhaps explaining why I.T., also expressing the "silent" phenotype, does not carry the amplification.

Once the H family was discovered, we were interested to find out whether the amplification of the "silent" human CHE gene is a unique

phenomenon, particularly since OP poisons are recently being exploited both as commonly used insecticides and as war agents (6). In view of the over-expression of BuChE that we observed in brain tumors (157) and the altered properties of BuChE in the serum of carcinoma patients (158), it is possible that the normal •CHE gene could also be subject to DNA amplification events. DNA transfection and transgenic mice experiments to examine this point are the logical route for continuation of these experiments. Finally, it would be important to determine whether repetitive exposure to organophosphates (6) provides a selective pressure for gene amplification at the CHE locus and whether other commonly used chemical agents have similar effects on additional loci in man and other species.

III.7. Correlation of cholinesterase gene amplification with hemocytopoiesis in vivo

To further search for CHE gene amplification, the postulated relationship between the family of ChE and hematopoietic commitment and differentiation was investigated using cDNA probes. These probes detected the presence of multiple copies of the genes coding for AChE and ChE in 25% of the leukemic DNA samples examined. Amplification of DNA sequences occurring at specific chromosomal breakpoints has been increasingly found in various malignancies (93). In several cases, these changes were correlated with cellular growth and development effects (159). One region that is conspicuously altered in leukemias appears on the long arm of chromosome No. 3 (90-92), where we recently mapped the CHE genes (85). In the mouse, ChE inhibitors and ACh analogs induce abnormal proliferation of megakaryocyte progenitor cells, both in vivo (88) and in vitro (89). Taken together, this appeared to be sufficient to initiate a search for structural changes within the ACHE and CHE genes in leukemias. Our finding of 6 of 20 amplification events among both genes, in cases of hematocytopoietic abnormalities, suggests that these apparently unrelated pieces of evidence might be connected.

The occurrence of these gene amplification events could reflect a specific origin of replication within the amplified CHE genes or in an adjacent oncogene (93,159). Yet another possibility is that of the insertion of a retroviral sequence, followed by the extension of its amplification into the chromosomal region of the CHE genes. The amplification of the CHE gene on chromosome No. 3 that we recently found in a family exposed to chronic doses of parathion, a potent ChE inhibitor (122), could be an example for the first option. It should be noted, however, that in that particular family the CHE gene was the only one to be amplified (Soreq, Prody and Zakut, unpublished observation). Other examples are the changes in the Ig genes close to the c-myc oncogene in Burkitt's lymphoma (160) and the amplification of cellular DNA sequences at the boundaries of the insertion site of polyoma DNA (161).

The co-amplification of the genes coding for both AChE and BuChE in our patients either indicates that these were co-localized at the same chromosomal region prior to the amplification event and were amplified together, or reflects the occurrence of recombination events

between these two genes during the amplification process. Alternatively, the ACHE and CHE genes might be independently subjected to the same selection pressure to be amplified. Amplification of various genes, including co-localized ones, has repeatedly been found in multidrug-resistant cell lines (162). Chromosomal rearrangement has also been proposed to facilitate gene amplification in drug-resistant cells by juxtaposing homologous segments (163). In Drosophila, high frequency of novel recombinational events was noted for the ace locus, carrying the structural ACHE gene (164). Precise mapping of the yet unlocalized gene(s) coding for human AChE will be required to clarify this issue.

Appearance of novel restriction fragments in the pronounced cases of the ACHE and CHE gene amplifications could be due to overlapping but nonequal regions of DNA having been amplified in the various individuals, perhaps reflecting variable origins of replication resulting from retroviral transposition. Various insertion sites for amplifiable retroviral sequences have been observed in the human genome, including a chromosome 3q site for leukemia virus sequences (165) close to the location of the CHE genes (85). Alternatively, the different patterns obtained in the various analyzed DNAs could reflect genetic alterations in the amplified genes, such as those observed for the amplified c-myc proto-oncogene in primary breast carcinomas (166) or those occurring in the dihydrofolate reductase gene in methotrexate-treated leukemic cells (80).

The possibility should be considered that the amplification of ChE-encoding genes was induced by continuous exposure to ChE inhibitors (i.e., OP agricultural insecticides, see III.6). The amplification of the ACHE and CHE genes in leukemias is not a random process, as it does not involve irrelevant sequences such as the ribosomal protein gene. CHE gene amplification could be advantageous to blood cells to which ChE activities are essential by creating acquired resistance to ChE inhibitors, like the amplification and over-expression of multidrug resistance genes (167) and the amplification of genes induced by arsenic (168). To further examine this possibility, the levels of expression of the amplified ACHE and CHE genes in hematocytopoietic disorders will have to be measured in individuals under chronic exposure to OP insecticides.

The putative involvement of ChEs in the etiology of hematocytopoietic disorders is of particular importance, in view of the multiple reports implicating these enzymes in growth and development. If ChEs are indeed important for hematocytopoiesis, the amplification of ChE-encoding genes would be analogous to other amplifications in malignancies. Examples include that of the genes coding for the epidermal growth factor receptor in malignant gliomas (169-171), the amplification of the neu oncogene in breast cancer, which is correlated with relapse and survival (172), and the amplification of N-myc in neuroblastoma, associated with the rate of progress of the disease (173). Although ChEs are not homologous to oncogenes, we have previously found altered modes of their expression in malignant gliomas (157) and more recently, in the sera of patients with various carcinomas (158). It would be interesting to reveal whether these reflect parallel amplification phenomena, giving multiple types of

tumor cells growth advantages.

IV. Methods

IV.1 cDNA libraries and screening

cDNA libraries prepared in the lambda gt11 vector were obtained from Dr. P. Lazarrini (Washington, D.C.) and were screened with selective probes as described (5). Positive clones were sequenced using the Amersham M13 sequencing kit following the enclosed instructions. Sequence alignment and analysis were performed on an IBM-XT personal computer using Beckman's Microgenie software. Dot matrix homology and hydrophobicity analyses were performed as previously described (5, 9, 43).

IV.2 In vitro transcription of SP6 ChEmRNA

The full-length cDNA coding for human BuChE (43) was inserted into the pSP64 transcription vector (Promega-Biotek) immediately downstream from the salmonella bacteriophage promoter (44). In vitro transcription and capping of pSP64-ChE constructs was essentially according to Krieg and Melton (44). Briefly, the reaction mixture contained 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM Spermidine, 0.01% BSA, 10 mM Dithiothreitol, 0.5 mM of ATP, CTP, UTP and GTP, 0.5 mM of m^7GpppG (Pharmacia), 100 ug/ml of pSP64-ChE DNA, 1 unit/ul of human placental RNase Inhibitor (Amerhsam) and 0.2 units/ul of SP6 RNA polymerase (Amersham). Incubation was at 37°C for 1.5 hr, after which a second equal portion of RNA polymerase was added for an additional 1.5 hr. Reaction was stopped by adding DPRF grade DNAse (Worthington) to a final concentration of 1 unit/ug DNA for 20 min at 37°C. RNA was extracted twice by phenol:chloroform: isoamyl alcohol and once with chloroform:isoamyl alcohol. RNA was ethanol-precipitated, pelleted by 10 min centrifugation in an Eppendorf centrifuge, washed with 70% ethanol and dissolved in sterile double-distilled water. Yields, as calculated by A_{260} (where 1 A_{260} = 40 ug of RNA) were ca. 40 ug full-length (2500 nucleotides) RNA chains per 20 ug of pSP64-ChE template.

IV.3 In ovo expression of human BuChE

All protocols for microinjection, homogenization, fractionation, and ChE activity assays have been described in detail (131,133). Briefly, stage 6 oocytes from mature frogs were surgically excised and manually teased from ovarian connective tissue; Capped, Poly (A)-tailed, synthetic BuChEmRNA was microinjected in 5 ng/oocyte quantitiies. In co-injection experiments, 25 ng poly $(A)^+$ RNA/oocyte was employed.

After an overnight incubation (16hr) at 20°C, the incubation medium was removed and the oocytes were homogenized in 150 ul/10 oocytes of fresh Barth's medium in a glass-teflon homogenizer. The total homogenate was centrifuged 10 minutes, 435,000 x g, 4°C in a Beckman TL100 centrifuge. The supernate was recovered and considered to reflect the soluble, cytoplasmic fraction. The pellet was resuspended in 150 ul of 10 mM Tris (pH 7.4), 1M NaCl, 1% Triton X-100, 1mM EDTA and spun for 5 minutes as above. The resultant supernatant was

recovered and considered to represent the detergent-extractable fraction. Samples were stored at -20°C.

Cholinesterase activity was measured by monitoring the release of thiocholine from thioester analogs of acetyl- and butyrylcholine in a colorimetric assay. Briefly, 5- to 10-ul samples were assayed in 200 ul final volume of 0.1M phosphate buffer (pH 7.0), 0.75 mM substrate (Iodide salt), 0.5 mM Dithionitrobenzoic acid. Assays were performed in 96-well microtiter plates and A_{405} was measured at regular intervals in an automatic microplate reader. Sucrose gradient analysis was performed as described (108,131).

IV.4 Immunohistochemistry

a. Antibodies

Protocols for immunohistochemistry have been published (133). Briefly, Rabbit polyclonal antibodies prepared against Torpedo electric organ AChE, recently shown to interact specifically with clone-produced human BuChE peptides (108), were gratefully received from Drs. S. Camp and P. Taylor (San Diego, California). The antibodies were preadsorbed for 2 hrs on non-injected oocyte sections to prevent cross-species interaction with the amphibian enzyme, and diluted 1:100 prior to incubation with injected oocyte sections.

b. Light microscopy analysis of oocyte sections

Twenty four hours after microinjection the oocytes were fixed for 2 hr in fresh 4% paraformaldehyde in 0.1 M phosphate buffer(pH 7.4). After incubation in graded sucrose (5%, 10%, 20% wt/vol in 100 mM phosphate buffer, pH 7.3), the oocytes were embedded, animal pole up, in O.C.T. compound (Tissue-teck), and frozen in 2-methylbutane cooled to -120°C with liquid nitrogen. 10-um sections were prepared in a cryotome at -25°C. The sections were collected on glass slides coated with 4% gelatin, washed twice in PBS/0.1 M glycine and incubated in PBS/4% goat serum. After an overnight incubation at 4°C in primary antibody diluted in PBS/ 4% goat serum, the sections were washed three times in PBS/goat serum and incubated with the fluorochrome-conjugated second antibody. After three further washes as above, the sections were mounted on coverslips in Gelmount (BIOMEDA) non-autofluorescent medium. The sections were observed using a LEITZ light-microscope.

In order to evaluate the non-specific binding of second antibody we have used a phycoprobe-linked second antibody (anti-rabbit IgG H+L BIOMEDA) which gives a yellow signal when specifically complexed with the first antibody and a green signal when non-specifically adsorbed to the oocyte section. The phycoprobe stock solution (1 mg/ml) was centrifuged five minutes at 12,000 x g, the supernatant was discarded, and the pellet was dissolved in 0.01M phosphate buffer (pH 7.0) to a final concentration of 10 ug/ml. Incubations and washes were performed as described above.

c. Electron microscopic techniques

Immunogold labeling was performed on 5-um thick cryostat sections. The same technique utilized for light microscopy was performed up to the first antibody incubation, but the PBS/goat serum was replaced by PBS/1% BSA. After three PBS/BSA washes, the sections were incubated with 5 nm colloidal gold particles conjugated to protein A (Janssen Life Sciences Products, Beese, Belgium) for 2 hr. The sections were washed three times with PBS and fixed in 2.5% glutaraldehyde/0.5% tannic acid in PBS for 1 hr at 4°C. After washing, the sections were postfixed in 2% osmium tetrodoxide in 0.1 M phosphate buffer(pH 7.0), for 30 min at 4°C, dehydrated and embedded in Epon. Ultrathin sections were cut, poststained 3 min in uranylacetate 50% in acetone and 2 min in 2% lead citrate, and then observed with a Philips EM 410 electron microscope.

IV.5 Cross immunoreactivity studies

Construction and expression of pEX-ChE plasmids, protein blot immuoreaction, elicitation and purification of rabbit anti-cloned-ChE antiserum, erythrocyte ghost preparation, sucrose gradient centrifugation, and in-situ immunochemistry to muscle tissue have been described in detail (108).

IV.6 Analyses of cross-homology between thyroglobulin and cholinesterase antibodies.

a. Patients

The groups studied were: 1) 9 patients with Graves' ophtalmopathy, at least grade 3 according to the American thyroid association classification and 2) 5 normal individuals. Igs were prepared by ammonium sulphate precipitation of whole sera.

b. Antigens

Human cholinesterase was used as a recombinant lysogenic protein produced by a clone isolated from a lambda gt10 library of human fetal brain mRNA as described (108). Human thyroglobulin, prepared from the thyroids of patient with Graves' disease by differential centrifugation, was passed through a Sepharose 4B column to remove immunoglobulin, and was gratefully received from Drs. Ludgate and Vassart, Brussels, together with polyclonal anti-thyroglobulin antibodies.

c. Dot blots

0.2 ug of bacterial extract containing the ChE protein was dotted onto nitrocellulose filters which were incubated in the first antibody at 50 ug/ml in PBS-milk mixutre at room temperature for 75 minutes. Filters were washed in PBS-milk and incubated with ^{125}I protein A (Amersham 5 x 10^5 cpm/ml, 35 uCi/mg) in PBS-milk, again for 75 min at room temperature. Blots were extensively washed and subjected to scintillation counting. Results are expressed as cpm after substracting the value obtained using non-recombinant plasmid

proteins. Immunoblots of gel electrophoresed proteins were performed as detailed above.

IV. In vivo CHE gene amplification measurements

Serum ChE activity measurements, DNA blot hybridization, and dot blots were performed as described (122, 43). In situ gene mapping by hybridization to spread chromosomes has been described in detail (85).

IV.8 Co-amplification in peripheral blood cells

Methods have been published (121). Briefly, blood samples were drawn in 13.3 mM EDTA (pH 7.5) from seven patients suffering abnormal platelet counts and leukemia. Blood DNA from 30 apparently healthy individuals served as controls. For hybridization experiments, ten micrograms of purified DNA were digested to completion with various restriction enzymes and electrophoretically separated on 1.2% horizontal agarose gels. DNA was transferred to GeneScreen membranes (NEN, DuPont). Filters were subjected to hybridization with [^{32}P]-labelled AChE or BuChE cDNA (5 X 10^9 dpm/ug). All other protocols were as previously described (122).

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